DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE AND TRIPROLIDINE HYDROCHLORIDE IN COUGH SYRUP FORMULATION

Dissertation

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MADURAI - 625 107, TAMIL NADU.

MAY – 2018

Certificate

This is to certify that the dissertation entitled **"DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE AND TRIPROLIDINE HYDROCHLORIDE IN COUGH SYRUP FORMULATION"** Submitted by **Ms. K. SUGANYA (Reg. No. 261630051)** in partial fulfillment of the requirements for the award of degree of **Master of Pharmacy** in **Pharmaceutical Analysis** under **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** Chennai, done at **K.M. COLLEGE OF PHARMACY,** Uthangudi**,** Madurai. It is a bonafide work carried out by them under my guidance and direct supervision during the academic year of 2017 - 2018. This dissertation partially or fully has not been submitted for any other degree or diploma of this university or other universities.

GUIDE **PRINCIPAL**

Mr. D. Shankar, M. Pharm., DSM., (Ph.D.),
Associate Professor & Research Coordinator,
Dept. of Pharmaceutical Analysis,
K.M. College of Pharmacy,
Uthangudi, Madurai - 625 107.
Tamil Nadu.

Dr. M. Sundarapandian, M. Pharm., Ph.D.,
Professor and HOD,
Dept. of Pharmaceutical Analysis,
K.M. College of Pharmacy,
Uthangudi, Madurai - 625 107.
Tamil Nadu.

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(K. SUGANYA)

DEDICATED

TO

MY BELOVED

PARENTS, GUIDE

& FRIENDS

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LIST OF ABBREVIATIONS

HPLC	:	High Performance Liquid Chromatography
LC	:	Liquid Chromatography
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
UV	:	Ultra Violet
MS	:	Mass Spectrometry
NMR	:	Nuclear Magnetic Resonance
IR	:	Infra Red
ESR	:	Electron Spin Resonance
ELSD	:	Evaporative Light Scattering Detector
RSD	:	Relative Standard Deviation
N	:	Number of Theoretical Plates
SD	:	Standard Deviation
NMT	:	Not More Then
NLT	:	Not Less Then
K'	:	Capacity Factor
R_S	:	Resolution
T	:	Tailing Factor
nm	:	Nanometer
mm	:	Millimeter
mg	:	Milligram
ml	:	Milliliter
mM	:	Millimolar
µl	:	Microliter

µg	:	Microgram
µm	:	Micrometer
µ	:	Micron
ºC	:	Degree Centigrade
RP	:	Reverse Phase
pH	:	Hydrogen ion Concentration
PDA	:	Photo Diode Array
t_R or **RT**	:	Retention Time
v_R	:	Retention Volume
S. No.	:	Serial Number
v/v	:	Volume/Volume
%	:	Percentage
Sec	:	Second
Min	:	Minute
RH	:	Relative Humidity
RI	:	Refractive Index
CP	:	Codeine Phosphate
TH	:	Triprolidine Hydrochloride
e.g.	:	Example
USP	:	United States of Pharmacopoeia
FDA	:	Food Drug Administration
API	:	Active Pharmaceutical Ingredient
ICH	:	International Conference of Hormonization

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CHAPTER - I

INTRODUCTION

1.1 Pharmaceutical Analysis:

- Pharmaceutical analysis is the branch of science which deals with identification of substances and determination of amount present in particular sample. Also pharmaceutical analysis deals with bulk materials, dosage forms and more recently, biological samples in support of Bio-pharmaceutical and Pharmacokinetic studies.
- Analysis can be divided into areas called qualitative and quantitative analysis. Pharmaceutical products synthesized and identified using Instrumental Techniques.[1] These methods are used extensively in the qualitative assurance of raw materials, in process quality assessment, stability of the drugs on storage monitoring drugs concentrations in various body fluids or tissues.

The types of analysis can be distinguished in two ways**:**

a. Qualitative Analysis:

To refer identity of product, i.e., it yields useful clue from which the molecular or atomic species, the structural features or the functional groups in the sample can be identified.

b. Quantitative Analysis:

To refer the purity of the product, i.e., the results are in the form of numerical data corresponding to the concentration of analytes.

1.2 Types of Analytical Methods:

The various methods of analysis [2] can be grouped into two categories namely

a. Chemical Methods

b. Instrumental Methods

a. Chemical Methods:

In these methods, volume and mass are used as means of detection.

- ❖ Titrimetrical methods like acid-base, oxidation-reduction, non-aqueous, complexometric and precipitation titrations
- ❖ Gravimetric and thermo gravimetric methods
- ❖ Volumetric methods

b. Instrumental Methods:

These methods are based on the measurement of specific and non-specific physical properties of substances. The Block diagram of analytical instruments [3] and different instrumental methods based on principle are shown in **Figure No. 1** and **Table No. 1** repectively.

Figure No. 1. Block Diagram of Analytical Instruments Showing the Stimulus and Measurements of Response

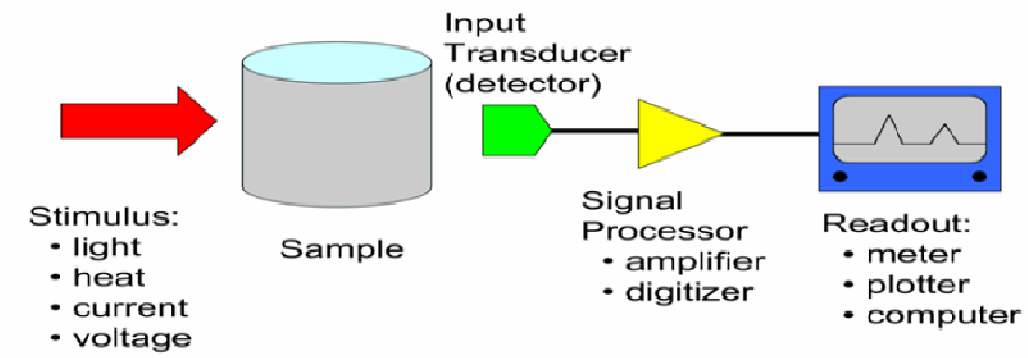


Table No. 1. List of Instrumental Methods Vs Principle

S. No.	Principle	Instrumental Methods
1	Emission of Radiation	X-ray Emission Spectrometry and Fluorescence Spectrometry
2	Absorption of Radiation	UV/Visible Spectrophotometry, NMR and IR Spectrometry, ESR Spectroscopy and Atomic Absorption Spectrometry
3	Mass to Charge Ratio	Mass Spectrometry (MS)
4	Refraction of Radiation	Refractometry
5	Scattering of Radiation	Nephelometry
6	Rotation of Radiation	Polarimetry
7	Electrical Potential	Potentiometry
8	Electrical Current	Amperometry and Polarography
9	Electrical Resistance	Conductometry
10	Thermal Properties	Differential Thermal Analysis, Differential Scanning Calorimetry and Thermogravimetry
11	Partition/Adsorption	Chromatographic Techniques

1.3 Chromatography: [4]

- Chromatography (from Greek chroma, color and graphein to write) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "Mobile Phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phase. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.
- Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the component of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture.
- The two are not mutually exclusive. The history of chromatography begins during the mid-19th century. Chromatography, literally "color writing", was used and named in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll.
- New type of chromatography developed during the 1930s And 1940s made the technique useful for many types of separation process. Some related techniques were developed during the 19th century (and even before), but the first true chromatography.

1.3a Chromatographic Terms:

- The ***analyte*** is the substance to be separated during chromatography.
- ***Analytical chromatography*** is used to determine the existence and possibly also the concentration of analyte (s) in a sample.
- A ***bonded phase*** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.
- A ***chromatogram*** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture. Plotted on the x-axis is the retention time and plotted on the y-axis a signal (for example obtained by a spectrophotometer, mass spectrometer or a variety of other detectors) corresponding to the response created by the analytics exiting the system. In the case of an optimal system the signal is proportional to the concentration of the specific analytes separated.
- A ***chromatograh*** is equipment that enables a sophisticated separation e.g. gas chromatographic or liquid chromatographic separation.
- ***Chromatography*** is a physical method of separation in which the compounds to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves in a definite direction.
- The ***eluent*** is the mobile phase leaving the column.
- An ***eluotropic*** series is a list of solvents ranked according to their eluting power.
- An ***immobilized phase*** is stationary phase which immobilized support particles, or on the inner wall of the column tubing.

- The ***mobile phase*** is the phase that moves in a definite direction. It may be a liquid (Liquid Chromatography, Capillary Electro Chromatography, Gas Chromatography and Supercritical Fluid Chromatography). The mobile phase consists of the sample being separated and the solvent that moves the sample through the column. In the case of HPLC the normal phase or polar solvents in reverse phase moves through the chromatography column where the sample interacts with the stationary phase and is separated.
- ***Preparative chromatography*** is used to purify sufficient quantities of a substance for further use, rather than analysis.
- The ***retention time*** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.
- The ***sample*** is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components.
- The ***solute*** refers to the sample components in partition chromatography.
- The ***solvent*** refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatograph.
- The ***stationary phase*** is the substance fixed in place for the chromatography procedure. Example includes the silica layer in thin layer chromatography.
- The ***detector*** refers to the instrument used for qualitative and quantitative detection of analytes after separation.

1.3b Types of Chromatography:

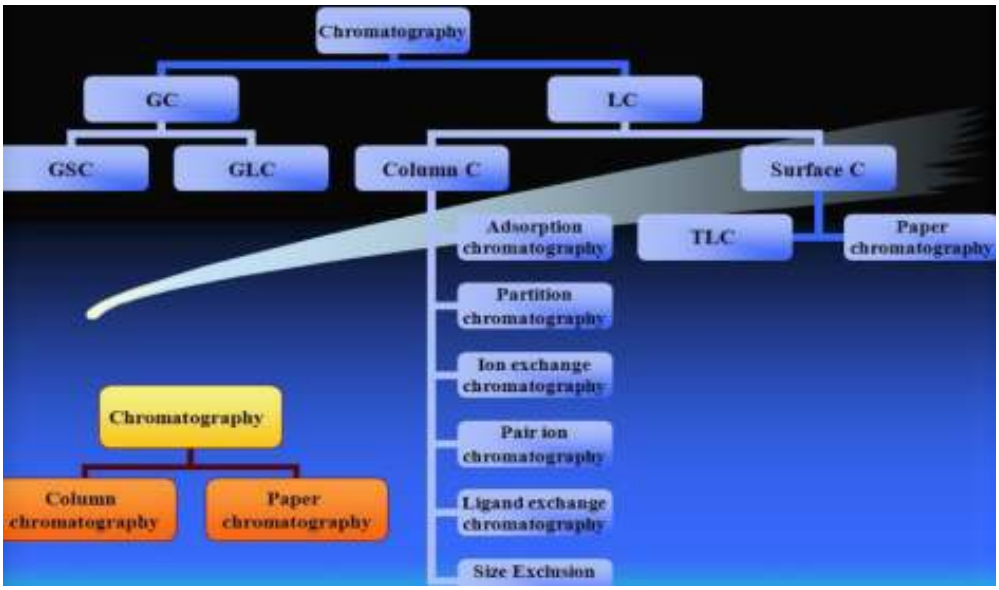
Chromatography can be classified into many types are shown in **Figure No. 2.**

- **Based upon the nature of stationary and mobile phase:**
  - Gas-Solid Chromatography
  - Gas-Liquid Chromatography
  - Solid-Liquid Chromatography
  - Liquid-Liquid Chromatography
- **Based on the principle of separation:**
  - Adsorption Chromatography

    e.g. Gas-Solid Chromatography, Thin Layer Chromatography, Column Chromatography and High Performance Liquid Chromatography (HPLC)
  - Partition Chromatography

    e.g. Gas-Liquid Chromatography, Paper Chromatography, Column Chromatography
- **Based on the modes of chromatography:**
  - Normal Phase Chromatography
  - Reverse Phase Chromatography
- **Other types of chromatography:**
  - Ion Exchange Chromatography
  - Gel Permeation Chromatography
  - Chiral Chromatography

Figure No. 2. Classification of Chromatography

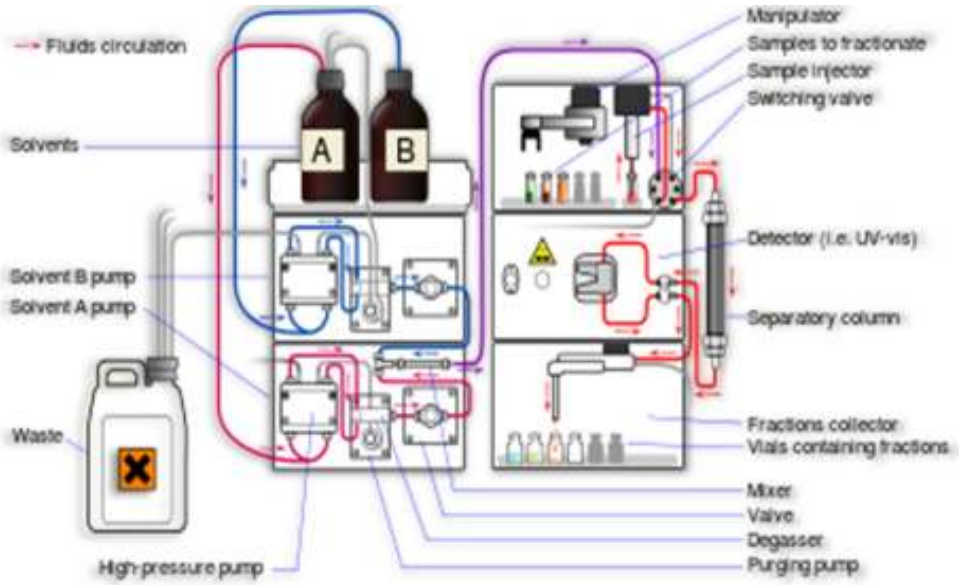


1.4 High Performance Liquid Chromatography (HPLC):

★ Initially, pressure was selected as the principal criterion of modern liquid chromatography and thus the name was "High Pressure Liquid Chromatography" or HPLC. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact, high performance is the result of many factors**:** Very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, and sensitive low volume detectors and, of course, good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase.

★ High Performance Liquid Chromatography (HPLC) is a versatile analytical technology widely used for the analysis of pharmaceuticals, biomolecules, polymers and many organic and ionic compounds. The preparative HPLC apparatus are shown in **Figure No. 3.**

Figure No. 3. Preparative HPLC Apparatus



1.4a Types of HPLC Techniques:

A) Based on Modes of Separation

- Normal Phase Chromatography
- Reversed Phase Chromatography

B) Based on Principle of Separation

- Adsorption Chromatography
- Ion Exchange Chromatography
- Size Exclusion or Gel Permeation Chromatography
- Affinity Chromatography
- Chiral Phase Chromatography

C) Based on Elution Techniques

- Isocratic Separation
- Gradient Separation

D) Based on the Scale of Operation

- ❖ Analytical HPLC
- ❖ Preparative HPLC

E) Based on the Type of Analysis

- ❖ Qualitative Analysis
- ❖ Quantitative Analysis

A) Based on Modes of Separation:

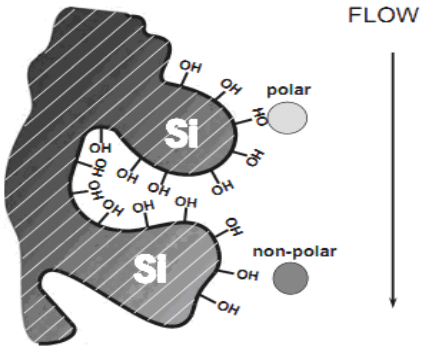
★ There are two modes of chromatography *viz*-normal phase mode and reverse phase mode. These modes are based on the polarity of stationary and mobile phase. Before explaining the modes, it is important to know the interactions which occur between solute, stationary and mobile phase.

- ✓ Polar-Polar - Interaction or affinity is more
- ✓ Nonpolar-Nonpolar - Interaction or affinity is more
- ✓ Polar-Nonpolar - Interaction or affinity is less

❖ **Normal Phase Chromatography:**

In normal phase mode, the stationary phase is polar in nature and the mobile phase is non polar **(Figure No. 4)**. In this technique, non-polar compounds travel faster and are eluted first. This is because of less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because of more affinity towards stationary phase and take more time to be eluted from the column. This is not advantageous in pharmaceutical applications since most of the drug molecules are polar in nature and takes longer time to be eluted and detected.

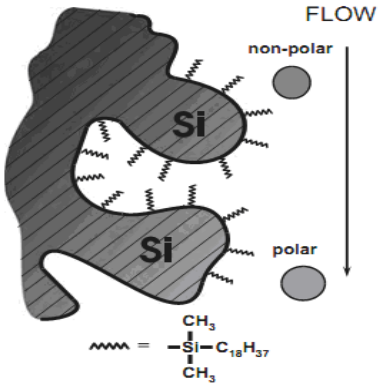
Figure No. 4. Normal Phase Chromatography



❖ **Reverse Phase Chromatography:**

In reverse phase mode, the stationary phase is non polar in nature and the mobile phase is polar **(Figure No. 5)**. Hence polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs are polar in nature, they are not retained for a longer time and eluted faster, which is advantageous.

Figure No. 5. Reverse Phase Chromatography



B) Based on Principle of Separation:

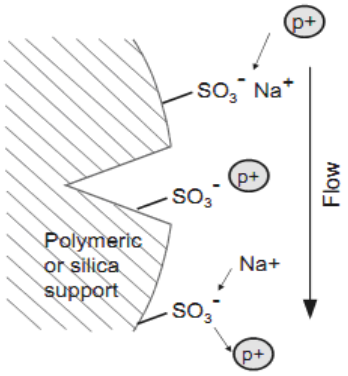
❖ **Adsorption Chromatography:**

The principle of separation is adsorption. Separation of components takes place because of the difference in affinity of compounds towards stationary phase.

❖ **Ion Exchange Chromatography:**

The principle of separation is ion exchange, which is reversible exchange of functional groups. In ion exchange resin is used to separate a mixture of similar charged ions. Typical stationary phases are cationic exchange (Sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials **(Figure No. 6)**. Mobile phases consist of buffers, often with increasing ionic strength, to force the migration of the analytes.

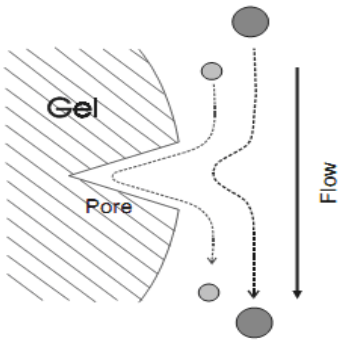
Figure No. 6. Ion Exchange Chromatography



❖ **Size Exclusion or Gel Permeation Chromatography:**

In this type of chromatography, a mixture of components with different molecular sizes is separated by using gels **(Figure No. 7)**.

Figure No. 7. Size Exclusion Chromatography



❖ **Affinity Chromatography:**

This uses the affinity of the sample with specific stationary phases. This technique is mostly used in the field of biotechnology, microbiology and biochemistry.

❖ **Chiral Phase Chromatography:**

This technique involves the separation of optical isomers can be done by using chiral stationary phases.

C) Based on Elution Techniques:

❖ **Isocratic Separation:**

In this the same mobile phase combination is used throughout the process of separation.

❖ **Gradient Separation:**

In this a mobile phase combination of lower polarity is used followed by gradually increasing the polarity.

D) Based on the Scale of Operation:

❖ **Analytical HPLC:**

Where only analysis of the samples can be done. Recovery of the samples for reusing is not done, since the sample used is very low.

❖ **Preparative HPLC:**

Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused.

E) Based on the Type of Analysis:

❖ **Qualitative Analysis:**

This is used to identify the compound, detect the presence of impurities, to find the number of components. This is done by using retention time values.

❖ **Quantitative Analysis:**

This is done to determine the quantity of the individual or several components in a mixture. This is done by comparing the peak area of the standard and sample.

1.4b Instrumentation of HPLC: [5]

❖ HPLC instrumentation includes a pump, injector, column, detector and data system **(Figure No. 8)**. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or Peak) on the recorder.

❖ Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computer, integrator and other data processing equipment are frequently used.

➧ ***The Main Components of HPLC are:***

a) Solvent Reservoir

b) Pump

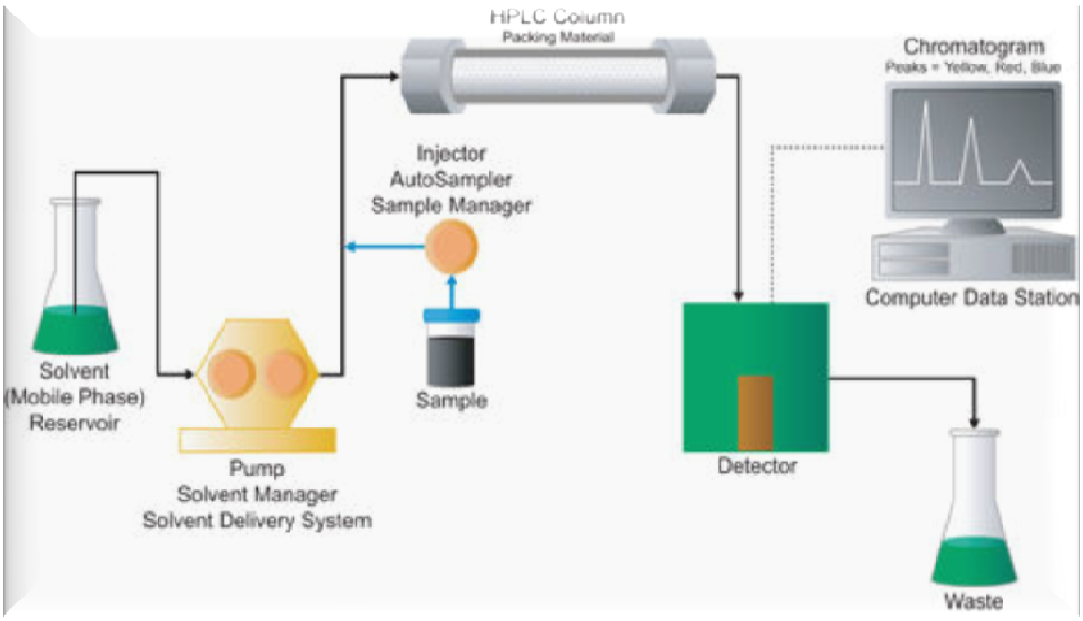
c) Injection Port

d) Column

e) Detector

f) Data Acquisition System

Figure No. 8. Components of High Performance Liquid Chromatography



a) Solvent Reservoir:

Solvent Reservoir is used to store mobile phase. Scott Duran bottles are commonly used as solvent reservoirs. The solvent reservoir must be made of inert material such as glass and must be smooth so as to avoid growth of microorganisms on its walls. It can be transparent or can be amber colored. A graduated bittle gives a rough estimate of mobile phase volume in the bottle. Solvent reservoirs are placed above HPLC system (at higher level) in a tray. They should never keep directly above the system as any spillage of solvent on the system may damage electronic parts of HPLC.

★ ***Mobile Phase:***

The power of HPLC in terms of being able to resolve many compounds is mainly due to the diversity of mobile phase or mobile solvents available. The mobile phase in HPLC, however, has a great influence on the retention of the solutes and the

separation of component mixtures. **Table No. 2** summarizes the commonly used HPLC solvents and their properties.

Table No. 2. Common HPLC Solvents and their Properties

Solvent	Solvent strength (E°)	bp (°C)	Viscosity (cP) at 20°C	UV cut-off (nm)	Refractive index
n-Hexane	0.01	69	0.31	190	1.37
Toluene	0.29	78	0.59	285	1.49
Methylene chloride	0.42	40	0.44	233	1.42
Tetrahydro-furan	0.45	66	0.55	212	1.41
Acetonitrile	0.55–0.65	82	0.37	190	1.34
2-Propanol	0.82	82	2.30	205	1.38
Methanol	0.95	65	0.54	205	1.33
Water	Large	100	1.00	<190	1.33

Ideally, solvents used as HPLC mobile phases should have these characteristics:

- High solubility for the sample components
- Non corrosive to HPLC system components
- High purity, low cost and UV transparency
- Other desirable characteristics include low viscosity, low toxicity and non flammability.

★ ***Buffers:***

The pH of the aqueous component in the mobile phase can have a dramatic effect on the retention of ionizable (acidic or basic) analytes. In RP-HPLC, the ionized form of the solute does not partition well into the hydrophobic stationary phase and has significantly lower pKa than the neutral form. Buffers are required to control the pH of the mobile phase. **Table No. 3** summarizes the commonly used HPLC buffers and their respective pKa and UV cut-off wavelength.

Table No. 3. Common HPLC Buffers and their Respective pKa and UV Cut-off Wavelength

Buffer	pK_a	UV cut-off (nm)
Trifluoroacetic acid*	0.3	210
Phosphate	2.1, 7.2, 12.3	190
Citrate	3.1, 4.7, 5.4	225
Formate*	3.8	200
Acetate*	4.8	205
Carbonate*	6.4, 10.3	200
Tris(hydroxymethyl) aminomethane	8.3	210
Ammonia*	9.2	200
Borate	9.2	190
Diethylamine	10.5	235

*Volatile buffer systems, which are MS-compatible.

b) Pump:

The HPLC pump is very important in component of the system. The pump delivers the constant flow of the mobile phase so that the separation of the components of the mixture occurs in a reasonable time. There are two types of pumping systems namely Isocratic and Gradient. The gradient type of pumps also classified into two types, those are:

- High pressure mixing, **e.g.** Mechanical and Piston pumps
- Low pressure mixing, **e.g.** Pneumatic and Quaternary pumps

Modern pumps have the following parameters:

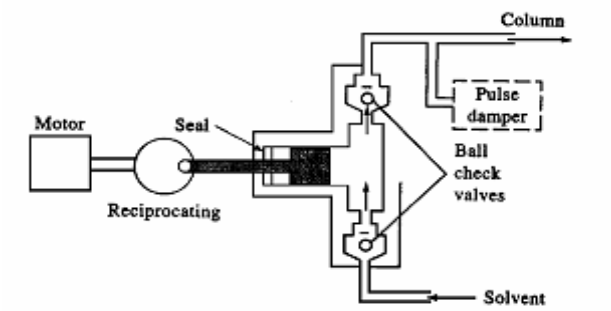
- Flow rate range**:** 0.01 to 5 ml/min
- Flow rate stability**:** NMT 1%
- For SEC flow rate stability should be less than 0.2%
- Maximum pressure**:** up to 3000 psi

There are three types of pumps commonly used:

★ ***Reciprocating Pump:***

Reciprocating pumps usually consist of a small chamber in which the solvent is pumped by the back and forth motion of a motor driven piston. Two check valves control the flow of solvent. Reciprocating pumps **(Figure No. 9)** have a disadvantage of producing pulsed flow, which must be damped as its presence is manifested as base line noise on the chromatogram. Advantages of this pump include their small internal volume, high output pressure, ready adaptability to gradient elution and independent of column backpressure and viscosity of solvent.

Figure No. 9. Reciprocating Pump



★ ***Displacement Pump:***

Displacement pumps usually consist of large syringe like chambers equipped with a plunger that is activated by a screw driven mechanism powered by stepping motor. Displacement pumps also produce a flow that tends to be independent of viscosity and backpressure. In addition, the output is pulse free. Disadvantages include limited solvent capacity (250 ml) and considerable inconvenience when solvents must be changed.

★ ***Pneumatic Pump:***

In pneumatic pumps, the mobile phase is contained in a collapsible container housed in a vessel that can be pressurized by a compressor gas. Pumps of this kind are

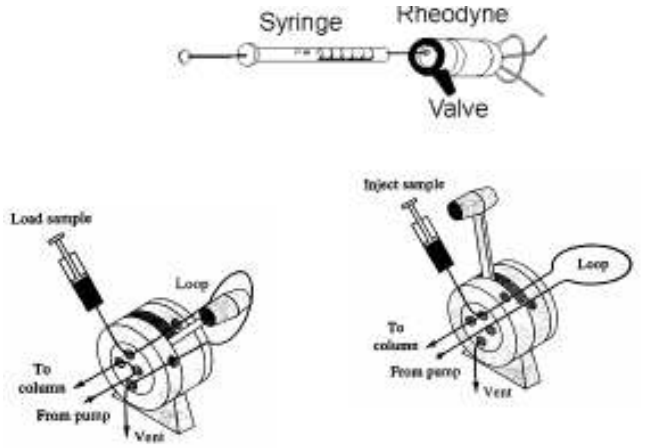
inexpensive and pulse free. They suffer from limited capacity, pressure output, dependence of flow rate on solvent viscosity and column backpressure. In addition, they are not amenable to gradient elution and are limited to pressures less than about 2000 psi.

c) Injection Port: [6]

- Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, and loss in efficiency or all of these.
- It is always best to remove particles from the sample by filtering over a 5 μm filter or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance.
- The most widely used sample injection system is loop injection valve. These valves provide precise injection volumes against high-pressures. In this sample load mode, the sample is flushed through the sample loop with the excess going to the drain. For sample is flushed through the sample loop with excess going to the drain. For sample injection, the valves are rotated so that the mobile phase flows through the sample loop flushing the sample to column. Precision of the sample injection using the loop injector may be as good as 0.1% RSD.

- In Rheodyne 7125 valve **(Figure No. 10)**, sample from a microlitre syringe is loaded into the needle port, filling the sample loop, which is a small piece of stainless steel tube connected between ports. Any excess goes to waste from another port. On turning to 'inject', the loop contents are flushed on to the column. A variety of loop volumes is available, commonly 10-50 µl.

Figure No. 10. Injector



d) Column: [7]

- Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns are usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed.
- Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment.

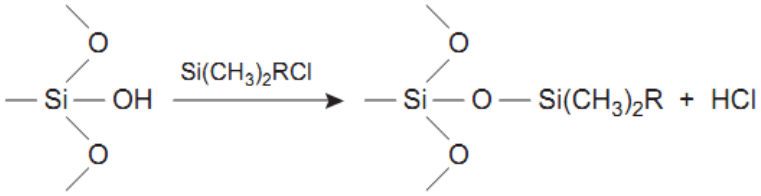
❖ In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for e.g. with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples.

There are three types of columns namely:

- Analytical columns
- Preparative columns
- Guard columns

Column Packing Materials (Adsorbents):

★ In liquid-liquid chromatography the stationary phase is a liquid film coated on a packing material consisting of 3-10 μm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to "bleed" from the column over time. To prevent this loss of stationary phase, it is covalently bound to the silica particles. Bonded stationary phases are attached by reacting the silica particles with an organochlorosilane of the general form $Si(CH_3)_2RCl$, where R is an alkyl or substituted alkyl group.



★ To prevent unwanted interactions between the solutes and any unreacted –SiOH groups, the silica frequently is "capped" by reacting it with $Si(CH_3)_3Cl$; such columns are designated as end-capped.

★ The properties of a stationary phase are determined by the nature of the organosilane alkyl group. If R is a polar functional group, then the stationary phase will be polar. Examples of polar stationary phases include those for which

R contains a cyano ($–C_2H_4CN$), diol ($–C_3H_6OCH_2CHOHCH_2OH$), or amino ($–C_3H_6NH_2$) functional group. Since the stationary phase is polar, the mobile phase is a nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called normal-phase chromatography.

★ In reverse-phase chromatography, which is the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group is an n-octyl (C8) or n-octyldecyl (C18) hydrocarbon chain. Most reverse-phase separations are carried out using a buffered aqueous solution as a polar mobile phase. Because the silica substrate is subject to hydrolysis in basic solutions, the pH of the mobile phase must be less than 7.5.

e) Detector: [8]

❖ Detectors detect various compounds as they elute out from column. The detector gives response in terms of a milivolt (mv) signal that is then processed by the computer (integrator) to obtain you a chromatogram. Basically detector consists of a flow cell through which the mobile phase and resolved. Sample moves optic shine through the detector cell and variation in optical properties are detected.

Various types of HPLC detector:

- UV-Visible detector
- Photo diode array detector (PDA)
- Fluorescence detector
- Conductometric and colorimetric detector
- Mass detector
- Evaporative light scattering detector (ELSD)

❖ Among these detector, photo diode array detector (PDA) is the most used detector in LC today. The PDA gives a three dimensional view of chromatogram (intensity Vs time) and spectra (intensity Vs wavelength) simultaneously. It can be called as Spectro- chromatogram. The detailed analysis of the data reveals more information on the complexity of coelution and helps in identifying the merged peaks and gives information on peak purity.

Ideal characteristics of a detector:

- Either is equally sensitive to all eluted peaks.
- The ideal detector give the response (area) proportional to the amount injected, irrespective of the size of sample.
- Cheap, reliable and easy to use.
- Should not be affected by change in temperature or mobile phase composition.
- It should be able to monitor small amount of compound.

f) Data Acquisition System:

★ Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The main goal is using electronic data system is to increase analysis accuracy and precision, while reducing operator attention.

★ There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent

processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Second, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimisation and deconvolution (i.e. resolution) of overlapping peaks. Finally, software safeguards can be designed to reduce accidental misuse of the system.

1.5 Chromatography Parameters: [9]

❖ ***Retention Time (t_R):***

The time between the sample injection and the peak maximum is called retention time. Retention time is measured in minutes or seconds.

❖ ***Retention Volume (v_R):***

Retention volume is the volume of mobile phase required to elute 50% of the component from the column. It is the product of retention time and flow rate.

$$\mathbf{v_R} = \text{Retention time } (\mathbf{t_R}) \text{ x Flow rate}$$

❖ ***Resolution (R_S):***

Resolution (R_s) is a measure of the degree of separation of two adjacent analytes. R_s is defined as the difference in retention time of the two peaks divided by the average peak width.

$$\text{Resolution, } R_s = \frac{t_{R2} - t_{R1}}{\left(\frac{w_{b1} + w_{b2}}{2}\right)} = \frac{\Delta t_R}{w_b}.$$

Where,

$\mathbf{t_{R1}}$ and $\mathbf{t_{R2}}$ = Retention time or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks

W_{b1} and W_{b2} = Respective peak widths determined at half peak height, measured in the same units as t_{R1} and t_{R2}

❖ ***Column Efficiency (N):***

It is called as the number of theoretical plates (N). It is a measure of the efficiency of the column. If the number of theoretical plates is high, the column is said to be highly efficient. If the number of theoretical plates is low, the column is said to be less efficient.

Number of theoretical plates, N = $\left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{4t_R}{W_b}\right)^2 = 16\left(\frac{t_R}{W_b}\right)^2$

❖ ***Tailing Factor (T):***

The tailing factor T, a measure of peak symmetry is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. In some cases, values less than one may be observed. As peak asymmetry increases integration and hence precision becomes less reliable.

$$\mathbf{T} = W_{0.05}/2f$$

Where,

$W_{0.05}$ = Width of peak at 5% height

f = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline

❖ ***Peak Symmetry:***

The peak symmetry can be represented in terms of peak asymmetry factor which can be calculated by using the following formula:

Peak asymmetry factor = B/A

Where,

B = The distance at 50% peak height between leading edge to the perpendicular drawn from the peak maxima

A = The width of the peak at half of the peak height

1.6 Method Development: [10]

- The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the exiting one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias.
- This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), Development of patient resistance and introduction of better drugs by competitors under this condition, standards and analytical procedures for this drug may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

There are several valid reasons for developing new methods of analysis:

- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing method may be too erroneous or unreliable.
- Existing method may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunity for improved methods, including improved analyte identification or detection limits, greater accuracy or precision or better return on investment.

- ❖ There may be a need for an alternative method for legal or scientific reasons, to confirm analytical data obtained by existing methods.

Goals for new or improved analytical method might include the following:

- ★ Qualitative identification of the specific analyte(s) of interest providing some structural information to confirm "general behavior" (**e.g.** retention time, colour change, pH etc).
- ★ Quantitative determination, at trace levels when necessary that is accurate, precise and reproducible in any laboratory setting when performed according to established procedures.
- ★ Ease of use, ability to be automated, high sample throughput and rapid sample turnaround time.
- ★ Decreased cost per analysis from using simple quality assurance and quality control procedures.
- ★ Sample preparations that minimize time, effort, materials and volume of sample consumed.

Steps Involved in Method Development:

Solubility Profile:

Solubility information in different solvents is useful while selecting the diluents for standard solutions and extraction solvents for test solutions.

Analytical Profile:

The spectral profile is useful in understanding the absorption characteristics, which helps in selection of detector and the wavelength for analysis.

Stability Profile:

The information on the stability of the drug substance with respect to storage condition is useful as it helps in adopting suitable and adequate precautions

while handling drug substance and its solutions.

- ***Selection and Optimization of Mobile Phase:***

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from analyte (API) peak. The selection of mobile phase is done always in combination with selection of column (stationary phase). The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

a) Buffer, if any and its strength

b) pH of the buffer or pH of the mobile phase

c) Mobile phase composition

a) Buffer, if any and its Strength:

Buffer and its strength play an important role in deciding the peak symmetries and separations. Various types of buffers can be employed for achieving the required separations. Some of the most commonly used buffers are:

- Phosphate buffers - KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, H_3PO_4 etc.,
- Acetate buffers - Ammonium acetate, Sodium acetate etc.,
- Triethylamine/Diethylamine buffers
- Buffers with various ion-pair reagents like Tetra butyl ammonium hydrogen sulphate

The retention time also depend on the molar strength of the buffer. Ideally, the strength of the buffers shall be opted from 0.05 M to 0.20 M.

The selection of buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be increased if necessary to achieve the required separations. But it is to be ensured that the higher buffer strengths shall not result in precipitations/turbidities either in mobile phase or in standard and test solutions while allowed to stand in bench top or in refrigerator.

b) pH of the Buffer or pH of the Mobile Phase:

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Depending on the pKa, drug molecules change retention. **e.g.** Acids show an increase in retention as the pH is reduced, while base show a decrease.

c) Mobile Phase Composition:

In reverse phase chromatography, the separation is mainly controlled by the hydrophobic interactions between drug molecules and the alkyl chains on the column packing material. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile.

- ***Selection of Column:***

Column plays the most important role in achieving the chromatographic separations. A column which separates all the impurities and degradants from API peak and which is rugged for variation in mobile phase shall be selected. Most chromatographic separations are achieved due to wide variety of columns available and due to flexibility to change and control each of the below parameters namely,

- Length and diameter of the column
- Pore volume
- Packing material
- Surface area
- Shape of the particles
- End capping
- Size of the particles
- % of carbon loading

- ***Selection of Flow Rate:***

Preferably the flow rate should NMT 2.5 ml/min. Check the ruggedness of the method by varying the flow rate by ±0.2 ml from the selected flow rate. Select the flow rate is based on the below parameters namely,

- ★ Retention time
- ★ Column back pressure
- ★ Separation of impurities
- ★ Peak symmetries

- ***Selection of Injection Volume:***

Generally an injection volume of 10 to 20 µL is recommended for the estimation of API. However, if the extractions are found to be difficult, then the injection volume can be increased up to 50 µL. But it is to be ensured that at the selected injection volume, the column is not overloaded, resolution between individual impurities from API peak and the peak symmetry are not compromise.

- ***Selection of Column Temperature:***

  Always it is preferable to optimize the chromatographic conditions with column temperature as ambient. However, if the peak symmetry could not be achieved by any combination of column and the mobile phase, then the column temperature above ambient can be adopted. The increase in column temperature generally will result in reduction in peak symmetry and peak retention. When found necessary, the column temperature between 30ºC and 80ºC shall be adopted. If a column temperature of above 80ºC is found to be necessary, packing materials, which can withstand to that temperature shall be chosen.

- ***Selection of Detector Wavelength:***

  Selection of detector wavelength is a critical step in finalization of the analytical method. In most cases HPLC method development is carried out with UV detection using either a variable-wavelength or a photodiode array detector, which can provide an adequate response for most samples.

- ***Establishment of System Suitability:***

  System suitability parameter has to be selected based on the tailing factor, resolution, plate count and percentage RSD.

1.7 Method Validation: [11, 12]

- Method validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".
- Method validation is an integral part of the method development. It is the process by which a method is tested by the developer or user for reliability, accuracy and

preciseness of its intended purpose and demonstrating that analytical procedures are suitable for their intended use that they support the identity, quality, purity and potency of the drug substances and drug products. Data thus generated become part of the methods validation package submitted to Center for Drug Evaluation and Research (CDER). Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

- Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations and throughout the life of the drug product. Data that are generated for acceptance, release and stability or pharmacokinetic will only be trustworthy if the methods used to generate the data are reliable. The process of validation and method design also should be clear in the development cycle before important data are generated. Validation should be on going in the form of re-validation with method changes.
- Though many types of HPLC techniques are available, the most commonly used method, the reversed-phase HPLC with UV detection, is selected to illustrate the parameters for validation. All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including

    A) System Suitability

    B) Accuracy

    C) Precision

    D) Specificity

    E) Linearity

F) Limit of Detection

G) Limit of Quantitation

H) Robustness

I) Ruggedness

A) System Suitability:

- According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed.
- System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns and analysts) is suitable for the intended application.
- System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (%RSD, retention time and area for six repetitions) are determined and compared against the specifications set for the method.
- These parameters are measured during the analysis of system suitability "sample" that is a mixture of main components and expected by-products. Below Table (**Table No. 4)** shows the list of parameters to be measured and their recommended limits obtained from the analysis of the system suitability sample as per current FDA guidelines on "Validation of Chromatographic Methods".

Table No. 4. System Suitability Parameters and Recommendations

S. No.	Parameters	Recommendations
1	Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k'>2.0
2	Repeatability	RSD $\leq$ 1% for N $\geq$ 5 is desirable
3	Relative Retention	Not essential as long as the resolution is stated
4	Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.
5	Tailing Factor (T)	T of $\leq$ 2
6	Theoretical Plates (N)	N > 2000

B) Accuracy:

- The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Accuracy can usually be determined in one of three ways:

- The procedure was applied to the known concentration of reference sample and the measured value to the true value was compared (defined by the organization, from which the sample received).

- The test results obtained were compared by the analytical procedure which was proved to be accurate with the results obtained from an existing alternate method that was known to be accurate.
- Spiking concept, by spiking either analyte/impurities into sample matrix with one another.

C) Precision:

❖ The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition. ICH has defined; precision may be considered at three levels namely repeatability, intermediate precision and reproducibility.

➢ ***Repeatability:***

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra- assay precision.

➢ ***Intermediate Precision:***

Intermediate precision was previously known as part of ruggedness. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

➢ ***Reproducibility:***

Reproducibility expresses the precision between laboratories as in collaborative studies. Multiple laboratories are desirable but not always attainable because of the size of the firm.

D) Specificity:

- The specificity of the method corresponds to the non-interaction of the placebo with that of the active particle. Typically these might include impurities, degradants, matrix, etc.

E) Linearity:

- A linear relationship should be evaluated across the range of the analytical procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content.
- If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

F) Limit of Detection (LOD):

- The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The detection limit is usually expressed as the concentration of the analyte (**e.g.** percentage or parts per million) in the sample and it can be calculated by using the following formula:

$$\textbf{LOD} = 3 \times S_a / b$$

Where,

S_a = Standard deviation of the intercept

b = Slope of the calibration curve

G) Limit of Quantitation (LOQ):

❖ The quantitation limit of an individual analytical procedure is the lowest amount of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is usually expressed as the concentration of the analyte (**e.g.** percentage or parts per million) in the sample and it can be calculated by using the following formula:

$$\mathbf{LOQ} = 10 \times S_a / b$$

Where,

S_a = Standard deviation of the intercept

b = Slope of the calibration curve

H) Robustness:

❖ The robustness of an analytical procedure is a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters such as flow rate, pH of the mobile phase, column temperature, percentage organic solvent strength and buffer concentration etc. It can be partly assured by good system suitability specifications.

❖ If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (**e.g.** Resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

I) Ruggedness:

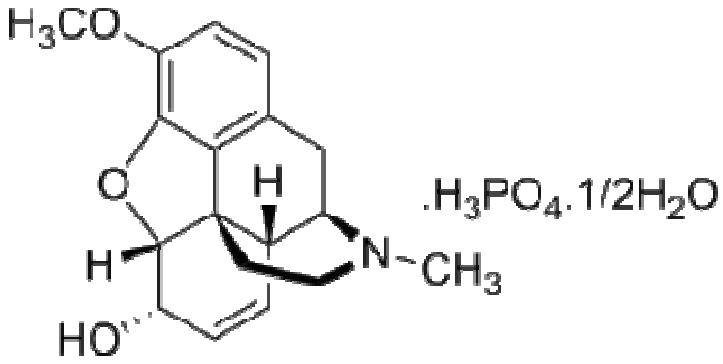
- Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals, solvents etc. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

CHAPTER - II

DRUG PROFILE

2.1 Codeine Phosphate: [13]

Structure:



CAS Number	**:**	76-57-3
Chemical Name	**:**	7,8-Didehydro-4,5 alpha-epoxy-3-methoxy-17-methyl morphinan-6 alpha-ol phosphate (1:1) (Salt) hemihydrates
Molecular Formula	**:**	$C_{18}H_{21}NO_3\ H_3PO_4\ ½H_2O$
Molecular Weight	**:**	406.4
Physical Properties	**:**	White crystalline powder, Odourless and Bitter taste
Solubility	**:**	Soluble in water
pK_a	**:**	8.21
Category	**:**	Antitussive and Analgesic
Dose	**:**	30 to 60 mg every 4 hours when necessary, to maximum of 200 mg daily

Mechanism of Action:

- Codeine phosphate is an opioid analgesic which binds with stereo specific receptors at many sites within the CNS to alter processes affecting both the perception of pain and the emotional response to pain. There are multiple sub types of opioid receptors, each mediating various therapeutic and/or side effects of drugs. Codeine has about one-sixth the analgesic activity of morphine.

Pharmacokinetics:

- Codeine is readily absorbed from the gastro-intestinal tract and metabolized by O-and N-demethylation in the liver to morphine and nor codeine which with codeine are excreted almost entirely by the kidney, mainly as conjugates with glucuronic acid.
- Most of the excretion products appear in the urine within 6 hours and 40 to 60 percent of the codeine is excreted free or conjugated, approximately 5 to 15 percent as free and conjugated morphine and about 10 to 20 percent as free and conjugated norcodeine.

Dose and Method of Administration:

- **Adults:** Codeine phosphate may be given orally in doses of 15 mg-60 mg every 4-6 hours as needed. It these doses fail to relive pain, larger doses rarely succeed and may give rise to restlessness and excitement. The maximum recommended daily dose is 300 mg.
- **Paediatric:** The usual paediatric dose for infants and children is 0.5 mg per kg of body weight or 15 mg per square metre of body surface, every 4 to 6 hours as needed. The maximum recommended dose is 240 mg in 24 hours. The duration of treatment should not normally exceed 3 days.

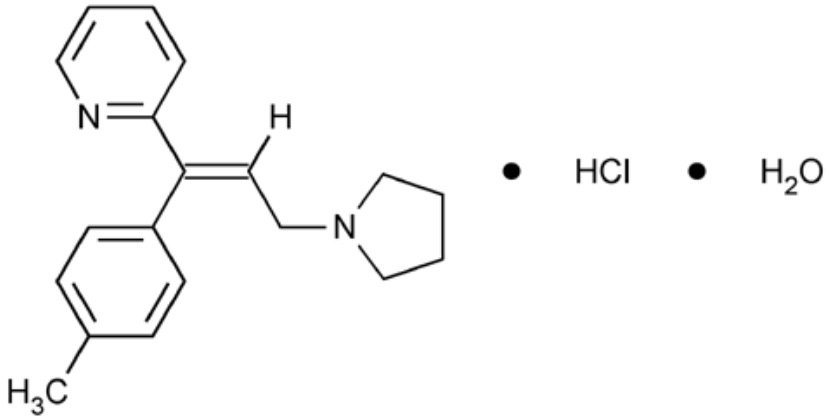
Side Effects:

Common side effects of Codeine phosphate include**:**

- Drowsiness, Rash
- Light headedness, Dependence
- Dizziness, Seizures
- Sedation, Confusion
- Shortness of breath
- Nausea, Near-fainting
- Vomiting
- Stomach pain
- Sweating
- Constipation
- Itching

2.2 Triprolidine Hydrochloride: [14]

Structure:



CAS Number	**:**	6138-79-0
Chemical Name	**:**	(*E*)-2-(3-pyrolidine-1-yl-1-(p-tolyl) prop-1-enyl pyridine hydrochloride monohydrate
Molecular Formula	**:**	$C_{19}H_{22}N_2$ HCl H_2O
Molecular Weight	**:**	332.9
Physical Properties	**:**	White crystalline powder, almost Odourless
Category	**:**	Histamine H_1-receptor antagonist
Dose	**:**	10-20 mg daily in divided doses

Mechanism of Action:

- Triprolidine hydrochloride exerts its action by acting as an H_1 receptor antagonist. It antagonizes most of the pharmacological actions of histamine and reduces allergic symptoms. It also has anticholinergic properties and reduces secretions.

Pharmacokinetics:

- Triprolidine hydrochloride is well absorbed orally. It is widely distributed in the body**.** It is mainly metabolized in the body by carboxylation and it is excreted through urine.

Side Effects:

Common side effects of Triprolidine hydrochloride include**:**

- Drowsiness
- Dizziness, Headache
- Nausea, Vomiting
- Loss of appetite
- Constipation
- Stomach upset
- Blurred vision
- Dry mouth / Throat

CHAPTER - III

LITERATURE REVIEW

- **Manassra *et al.,*** [15] proposed Simultaneous HPLC analysis of Pseudophedrine hydrochloride, Codeine phosphate and Triprolidine hydrochloride in liquid dosage forms. C18 column (250 x 4.0 mm) is used as the stationary phase with a mixture of Methanol:Acetate buffer:Acetonitrile (85:5:10, v/v) as the mobile phase. The factors affecting column separations of the analytes were studied. The calibration graphs exhibited a linear concentration range of 0.06-1.0 mg/ml for Pseudoephedrine hydrochloride, 0.02-1.0 mg/ml for Codeine phosphate and 0.0025-1.0 mg/ml for Triprolidine hydrochloride for a sample size of 5 µl with correlation coefficients of better then 0.999 for all active ingredients studied. The results demonstrate that this method is reliable, reproducible and suitable for routine use.
- **Capella-Peiro *et al.,*** [16] optimized Capillary Zone Electrophoresis method to quantitatively determine Codeine and Paracetamol via Central composite factorial design. Optimum separation conditions were achieved using Phosphate buffer 20 mm (pH 6.8) and voltage (15 kv). The optimized Procedure easily determination Codeine and Paracetamol with separation in less then 3 min. Calibration curves (R>0.999) were prepared, with LODs of 13.5 and 340 mg/ml for Codeine and Paracetamol respectively and a good RSD % (<3%). This method was applied to determine Codeine and Paracetamol in pharmaceutical formulations; recoveries coincided with stated contents.
- **Gomez *et al.,*** [17] described a simple, accurate and rapid method for the separation and Simultaneous determination of Codeine, Diphenhydramine, Ephedrine and

Noscapine present in cough-cold syrup formulations by Capillary Zone Electrophoresis. Separations were carried out in less then 10 min with a 20 mm Sodium tetra borate buffer, pH 8.5, the carrier electrolyte gave baseline separation with good resolution, great reproducibility and accuracy.

- **Kartal *et al.,*** [18] proposed LC method for the analysis of Paracetamol, Caffeine and Codeine phosphate in pharmaceutical preparation. Paracetamol, Caffeine and Codeine phosphate were separated using a μ bond pack C8 column by isocratic elution with flow rate 1.0 ml/min. the mobile phase composition was 420/20/30/30 (v/v/v/v) 0.01m KH_2PO_4, Methanol, Acetonitrile, Isopropyl alcohol and Spectrophotometric detection was carried out at 215 nm. The linear range of detection for Paracetamol, Caffeine and Codeine phosphate was between 0.400 and 1500 μg/ml; 0.075 and 90 μg/ml; 0.300 and 30 μg/ml respectively. The method has been shown to be linear, reproducible, specific, sensitive and rugged.
- **Hood *et al.,*** [19] developed a simple, accurate and precise RP-HPLC method for rapid and Simultaneous analysis of Codeine phosphate, Ephedrine HCl and Chlorpheniramine maleate in a cough-cold syrup formulation. Separations were carried out on a zorbax XDB C8 column (150 mm ID), 5 μm particle size. A gradient elution system was developed using varying percentages of two mobile phases: Methanol:Glacial acetic acid:Triethylamine (980:15:5 v/v) and Water:Glacial acetic acid:Triethylamine (980:15:5 v/v) with run time in less than 7 min with a flow rate of 1.5 ml/min and detected at a wavelength of 254 nm. The method was validated and met all analysis requirements of quality assurance and quality control recommended by FDA of the USA.
- **Ragonese *et al.,*** [20] described full and fractionated experimental designs for Robustness Testing in the High Performance Liquid Chromatographic Analysis.

A full factorial design relies on fewer assumptions and hence could be used to evaluate the effectiveness of the saturated design. Both designs were used to test a gradient HPLC method for the assay of Codeine phosphate, Pseudoephedrine hydrochloride and Chlorpheniramine maleate. Six HPLC conditions, including Wavelength, mobile phase pH and ion pairing reagent concentration were tested using the saturated design. One interaction effect was indicated as a confounding effect by the saturated design and this was confirmed by the calculating of the robust under the variety of HPLC conditions tested.

- **Lau *et al.,*** [21] proposed HPLC method using indirect conductometric detection for the Simultaneous determination of eight active ingredients in cough-cold syrups. It involves the use of an ultra here 5 µm spherical 80 A^o pore Cyano analytical column (250 x 4.6 mm) as the stationary phase with a mixture of Water, Acetonitrile and Ethanol (38:60:2) containing 1mm Perchloric acid as the mobile phase. The active ingredients included Bromhexine hydrochloride, Chlopheniramine maleate, Codeine phosphate, Dextromethorphan hydrobromide, Diphenhydramine hydrochloride, Ephedrine hydrochloride, Papaverine hydrochloride and Phenylephrine hydrochloride.
- **Weingarten *et al.,*** [22] developed a rapid, reliable and rugged assay for determining Codeine in human plasma using Reverse Phase HPLC with Fluorescence detection. This analytical method utilized an ion-exchange/ mixed-mode solid phase extraction procedure. The chromatographic separation was achieved using a 150 x 4.6 mm ID, 3 µm Reversed Phase C8 column at ambient temperature. Fluorescence detection (excitation at 214 nm and emission above 345 nm) for Codeine and Nalorphine allowed for a detectable limit of 5 µg/ml. The results showed that the method was linear from 10 to 300 ng/ml. The method

had good reproducibility, precision, accuracy and recoveries of 91 and 90% for Codeine and Nalorphine respectively. This method has been applied to study the pharmacokinetics of Codeine in normal human subjects.

- **Santoni *et al.,*** [23] developed a Reverse Phase HPLC method for the Simultaneous determination of Aspirin, Codeine phosphate and Propylphenazone in analgesic tablet formulation. The proposed method is also suitable for the determination of small quantities of Salicylic acid. The elution was isocratic using two C8 column and Methanol:Water (45:55) as mobile phase with 1.4% Acetic acid and 5 mm Tetramethyl ammonium bromide.
- **Chen *et al.,*** [24] developed a novel HPLC method for the Simultaneous determination of Codeine, Norcodeine and Morphine in plasma and urine. The compounds were separated on a cyano column (15 cm x 4.6 mm, 5 µm particle size) using a mobile phase of Acetonitrile-Triethylamine-Distilled water (4:0.1:95.9, v/v) pH 3.1 and then determined by Fluorescence detection. Calibration curve in the range 5-200 ng/ml for plasma and 0.1-10 µg/ml for urine were linear and passed through the origin. The imprecision and inaccuracy of the assay were less then 10% and the limits of detection were 2 µg/ml for all three compounds in human plasma.
- **Ginman *et al.,*** [25] described a procedure for the Simultaneous determination of Codeine and Ibuprofen in human plasma by HPLC with Fluorescence detector. The Codeine was first extracted from alkalinized plasma with Hexane: Dichloromethane (2:1, v/v) and then washed with sodium hydroxide solution. The Ibuprofen was then extracted with hexane from the plasma acidified with sulphuric acid. The organic layers were collected, evaporated to dryness and the

reconstituted residue was subjected to HPLC. The detection limit for codeine was 8 µg and for Ibuprofen 1mg.

- **Davidson *et al.,*** [26] described Spectrophotometric procedures for the assay of Triprolidine hydrochloride, Pseudoephedrine hydrochloride and Dextromethorphan hydrobromide in Actifed formulation. Triprolidine is assayed by the measurement of the difference absorbance at 301 nm between equimolar solutions of the sample extract in 0.1 M sulphuric acid and 0.1 M sodium hydroxide in ethanol (20% v/v). Dextromethorphan and Pseudoephedrine are assayed by measurement of the amplitudes in the second and fourth derivative spectra of the difference absorption spectrum of the sample solution. The measured values are proportional to the concentration of the drugs. The accuracy, precision and selectivity of the procedures are discussed. Applications of the assay are described for Actifed syrup and Actifed tablets.
- **El-Gindy *et al.,*** [27] developed HPLC method for the analysis of Paracetamol, Pseudoephedrine, Triprolidine, Methylparaben, Propylparaben, Sodium benzoate and their related substances including p-Aminophenol, Triprolidine Z-isomer, 4-hydroxy benzoic acid, and 4-chloroacetanilide in pharmaceutical syrup using C18 column at 25ºC with UV detection at 214 nm. A linear gradient elution was employed starting with 100% mobile phase A and 0% mobile phase B for 5 min to reach 40% mobile phase B at 16 min then 0% mobile phase A and 100% mobile phase B at 28 min. The total run time is 30 min using solution of 28 mM Sodium dehydrogenate phosphate containing 2.6 mM Hexane sulphonic acid sodium salt and adjusted to apparent pH 3.0 with Phosphoric acid-Acetonitrile in ratios of (90:10 v/v) and (60:40 v/v) as mobile phase A and mobile phase B respectively. All the mentioned compounds have been successfully separated and

quantified using the developed method. The developed method was linear with (r=0.9999) for all compounds. The proposed method was completely validated.

- **Sriphong *et al.,*** [28] developed Spectrophotometric method for Simultaneous Quantification of Triprolidine hydrochloride and Pseudoephedrine hydrochloride using second derivative method (zero-crossing technique). The second derivative amplitudes of Pseudoephedrine hydrochloride and Triprolidine hydrochloride were measured at 271 and 321 nm, respectively. The calibration curves were linear in the range of 200 to 1000 µg/ml for Pseudoephedrine hydrochloride and 10 to 50 µg/ml for Triprolidine hydrochloride. The method was validated for specificity, accuracy, precision, limit of detection and limit of quantitation. The proposed method was applied to the assaying and dissolution of Pseudoephedrine hydrochloride and Triprolidine hydrochloride in commercial tablets without any chemical separation. The results were compared with those obtained by the official USP method and statistical tests showed that there is no significant between the methods at 95% confidence level. The proposed method is simple, rapid and suitable for the routine quality control application.
- **Caglar *et al.,*** [29] developed a simple Reverse Phase HPLC method for Simultaneous determination of Pseudoephedrine hydrochloride, Pheniramine maleate, Acetaminophen, Guaifenisin, Pyrilamine maleate, Chlorpheniramine maleate, Triprolidine hydrochloride, Dextromethorphan hydrobromide and Diphenhydramine hydrochloride in cough and cold pharmaceuticals. The separation of these compounds was achieved within 37.9 min on a nucleolus gravity C18 column (250 x 4.0 mm, 5 µm). The chromatographic separation of these compounds performed in a single run by using isocratic mobile phase consisting of Methanol:Buffer mixture (38:62, v/v) at roomtemperature, with

flow rate of 0.75 ml/min. An Ultraviolet absorption at 210 nm was monitored. 2,4,6-Trimethoxy benzaldehyde was used as an internal standard. The selectivity, linearity of calibration, accuracy, interday and intraday precision and forced degradation studies were examined as parts of the method validation. The concentration-response relationship was linear over a concentration range of 0.2-250 µg/ml for Acetaminophen, 0.5-250 µg/ml for Pseudoephedrine hydrochloride and Pheniramine maleate, 1-250 µg/ml for Guaifenisin, 2.5-250 µg/ml for Chlorpheniramine maleate and Triprolidine hydrochloride, 5-250 µg/ml for Pyrilamine maleate and Diphenhydramine hydrochloride, 10-20 µg/ml for dextromethorphan hydrobromide with correlation coefficients better then 0.9993. The relative standard deviations of the intraday and intraday were all less than 4%.

- **De Orsi *et al.,*** [30] developed a simple, rapid and specific HPLC method for the Simultaneous determination of Triprolidine, Pseudoephedrine, Paracetamol and Dextromethorphen, in combination and in different pharmaceutical dosage forms using a Reverse Phase C18 column, gradient elution and UV detection at 254 and 280 nm. No preliminary extraction procedure is required for liquid formulation and a very simple extraction procedure is required for tablets and creams. The recovery of the drugs ranged from 96.0 to 98.7%. The assay results obtained for eight commercially available formulations were in agreement with the amounts declared. The linearity and precision of the method have been assessed.
- **Mallu *et al.,*** [31] developed for the determination of ten active Ingredients (Codeine phosphate, Paracetamol, Chloropheniramine maleate, Theophylline, Pseudoepidrine hydrochloride, Ambroxol, Salbutamol, Guaiphenesin, Dextromethorphan and Diphenhydramine hydrochloride) in all pharmaceutical

dosage forms, along with preservative (Sodium benzoate) and validated the method as per ICH and FDA guidelines. The separation was achieved on a X-terra C18 column (15 cm x 4.6 mm, 3.5 µm in the simple gradient mode using Sol-A; Buffer and Sol-B: Acetonitrile (0.5 min, Sol-A: 97-97; 5-10 min, Sol-A: 97-92; 10-15 min, Sol-A: 92-68; 15-23 min, Sol-A: 68-68; 23-25 min, Sol-A: 68-97 and 25-30 min, Sol-A: 97-97) with 0.8 ml per min flow rate. Column oven temperature maintained at 40 ºC and performed the analysis with 220 nm. Quantification was achieved with 40 µg per ml for all ingredients with 100 ± 3.0% recoveries. The method was validated by determining its sensitivity, linearity, accuracy and precision. The proposed method is single, shorter runtime, accurate and reproducible. This method can be applied for routine analysis of all ten active ingredients quantification in all pharmaceutical dosage forms.

- **Paidipala *et al.,*** [32] described a simple, selective, sensitive and precise Simultaneous HPLC analysis of tablets containing Dextromethorphan hydrobromide, Phenylephrine hydrochloride and Triprolidine hydrochloride. Good chromatographic separation was achieved using a kromasil C18 (250 x 4.6 mm, 5 µm) and mobile phase consisting of Methanol:Acetonitrile:0.1M Potassium dihydrogen orthophosphate buffer (75:15:10), adjusted to pH 6.8 with sodium hydroxide, at flow rate 1 ml/min. the PDA detector was used. The retention time of Dextromethorphan hydrobromide, Phenylephrine hydrochloride and Triprolidine hydrochloride were measured at 2.547, 3.783 and 6.017 min, respectively. The linear ranges for Dextromethorphan hydrobromide, Phenylephrine and Triprolidine hydrochloride were 48-112, 24-56 and 16-14 µg/ml, respectively. The recoveries of Dextromethorphen hydrobromide and Phenylephrine hydrochloride and Triprolidine hydrochloride in pharmaceutical

preparation were all greater than 98% and their relative standard deviations were NMT 2.0%. The limit of detection was 3.71, 1.90 and 0.52 µg /ml. The proposed method can be effectively applied for the Simultaneous estimation of three drugs in bulk and in combined dosage form.

- **Hinge *et al.,*** [33] developed First order derivative Spectrophotometric and HPLC methods for the determination of Triprolidine and Pseudoephedrine hydrochloride in tablet dosage form. In UV Spectrophotometric method, estimation of Triprolidine and Pseudoephedrine hydrochloride was carried out at the wavelength selected 246.20 nm and 263.50 nm for first order derivative method. Calibration curves were linear in the range of 2-10 µg/ml for Triprolidine and 48-240 µg/ml for Pseudoephedrine hydrochloride in derivative method. Correlation coefficient found to be close to 0.9950 for both the drugs. Accuracy for both the drugs was in the range of 99-101.5%. A simple liquid chromatography assay has been developed for the determination of Triprolidine and Pseudoephedrine hydrochloride. A column of C18 (250 x 4.6 mm, 5 µm) was used with a mobile phase consisting of Methanol:Water (80:20 v/v) at a flow rate of 1.0 ml/min and pH also adjusted to 3.0 with Orthophosphoric acid. Quantitation was achieved with UV detection at 246.20 nm based on the peak height ratios. Beer's law was obeyed in a concentration range of 5-25 µg/ml for Triprolidine and 120-600 µg/ml for Pseudoephedrine hydrochloride.
- **Abu Reid *et al.,*** [34] developed a Spectrophotometric method for the Simultaneous determination of Pseudoephedrine HCl (PSE) and Triprolidine HCl (TRI) in bulk and dosage forms. The method involved the determination of Pseudoephedrine in the presence of Triprolidine using two wavelengths (257 nm & 290 nm). Beer's law was obeyed in the concentration (152-760 µg/ml) and (6.4-32 µg/ml) with

good linearity (0.9996 and 0.9996) for Pseudoephedrine and Triprolidine respectively. The accuracy and the precision of the developed method were very good (RSD < 2%). The validity of the proposed method was confirmed through the statistical comparison of the obtained data with those of the official USP method.

- **Arif Aziz *et al.,*** [35] developed for the Simultaneous HPLC determination of Paracetamol with a various range of API'S (Active Pharmaceutical Ingredients) like Caffeine, Phenobarbital, Acetyl salicylic acid, Phenobarbital, Phenylephrine hydrochloride and Diphenhydramine hydrochloride, Salicylamide, Guaifenesin hydrochloride, Chlorpheniramine maleate, and Promethazine hydrochloride and excipients like Sodium benzoate, Propyl paraben, Para aminophenol, 4-Chloracetanilide and Methyl paraben in different dosage forms. Six methods are reviewed and discussed for the Simultaneous determination of Paracetamol. First method was related to HPLC chromatographic assay of Caffeine, Acetyl salicylic acid, Paracetamol, Phenobarbital in tablet formulation is performed using RP-HPLC technique with column C18, mobile phase Acetonitrile and Water with adjusted pH 2.5 having flow rate 2.0 ml/min at 207 nm. Second method was the assay of Paracetamol and Acelofenac, a RP-HPLC method at 265 nm using Hichrome C18, mobile phase Acetonitrile and Phosphate buffer having 0.8 ml/min. Third method was the estimation of Triprolidine, Pseudoephedrine, Dextrometherphan and Paracetamol where chromatography is carried put through C18 RP-HPLC technique first at 254 nm then 280 nm. Fourth method was for the Ibuprofen and Paracetamol having system parameter comprises of C18 column with eluent pH 7 (Acetonitrile and Phosphate buffer) and elution at 8.0 ml/min. Peaks were detected through UV detector 260 nm.

Fifth method was for Paracetamol and Camylofin dihydrochloride with C18 column at 220 nm using Methyl paraben as Internal standard. Last method but no least was done through gradient system at 215 nm with 40 mins run time. These six assays procedures can be used in any pharmaceutical lab the analysis of Paracetamol Combination in tablet, syrup, suspension and bulk.

- **Ma *et al.,*** [36] developed a new Chemiluminescence method with flow Injection analysis for the determination of Triprolidine hydrochloride. It was based upon the significant enhancing effect of Triprolidine Hydrochloride on the Chemiluminescence reaction of N-bromo succinimide with Luminal in presence of gold nanoparticles as a catalyst. Under the optimum conditions, the relative Chemiluminescence intensity was linearly related to the concentration of Triprolidine hydrochloride in the range of 1.0 -750.0 µg/ml. The detection limit (3Sb/S) was 0.2 µg/L and the relative standard deviation was 1.2% for 0.1 mg/L Triprolidine hydrochloride solution (n=11). The method was applied to the determination of Triprolidine hydrochloride in capsules and the study of interactions of Triprolidine hydrochloride with serum albumins.
- **Vijai Anand *et al.,*** [37] developed a simple, specific, accurate and stability indicating RP-HPLC method for the Simultaneous determination of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in cough syrup formulation using Zodiac C18, 3.5 µ, 150 x 4.6 mm column eluted with Solvent-A: Phosphate buffer (pH-2.3), Solvent-B: Acetonitrile by gradient elution pattern at a flow rate of 1.5 ml/min and a detection wavelength of 254 nm with injection volume of 50 µl at ambient (30ºC) temperature afforded the best separation of these analytes. The retention time of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate were found to be 7.18 min, 9.46

min and 10.86 min respectively. The system precision of this method was evaluated by calculating the %RSD of the peak areas of six replicate injections of the standard solution, which were found to be 0.41%, 0.35% and 0.37%. Accuracy studies were performed where the % recovery of three drugs was found to be 98.7%, 98.5% and 99.1% respectively. Linearity was established for Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in the range of 12.5-75 µg/ml, 5-30 µg/ml and 12.5-75 µg/ml respectively. Specificity of the current methods was demonstrated by good separation of the three analytes a small change in the following chromatographic parameters: Flow rate: 1.4 and 1.6 ml/min instead of 1.5 ml/min and pH of the buffer preparation in mobile phase: 2.2 and 2.4 instead of 2.3. The %RSD's were found to be within the limits. Ruggedness was demonstrated by analyzing three samples (assay) of syrup formulation by two analysts in the same laboratory on to different days. The %RSD values for the 12 samples are calculated to be guidelines and shown to be accurate, precise and specific. This method is agreeable to the routine analysis and can be successfully employed for simultaneous quantitative analysis of Codeine phosphate, Chlorpheniramine malealte and Sodium benzoate respectively in bulk drugs and formulations.

- **Kazemi *et al.,*** [38] studied a sensitive method for the determination of Codeine phosphate in water samples using Dispersive Liquid-Liquid Microextraction coupled with UV spectrophotometry. Parameters that affect on the extraction efficiency, such as kind and volume of the extraction and disperser solvent, extraction time, salt addition were investigated and optimized. Under the optimal conditions, the linearity of the method was obtained in the range 0.005-10 µg/ml with coefficient of (r^2) 0.9996.

- **Geetha Lakshmi *et al.,*** [39] developed and validated an accurate, sensitive, precise and robust RP-HPLC method for Simultaneous estimation of Codeine phosphate, Chlorpheniramine maleate and its Preservative in syrup formulation. Chromatographic separation was conducted on zodiac C18 (150 x 4.6 mm, 3.5 µm) column at ambient temperature using phosphate buffer (pH adjusted to 2.3 with Ortho phosphoric acid), Acetonitrile in gradient mode at a flow rate of 1.5 ml/min, while UV detection was performed at 254 nm. The retention time for Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate was found to be 7.169, 9.480 and 10.860 respectively. The method was found to be linear in the 12.5-75 µg/ml for Codeine phosphate, 5-30 µg/ml for Chlorpheniramine maleate and Sodium benzoate was found to be 98.7%, 98.5% and 99.1%, respectively. The developed method was validated in terms of accuracy, specificity, robustness, Precision and ruggedness. This method can be successfully used for the estimation of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in bulk and syrup formulations.
- **Nazir *et al.,*** [40] developed and validated for a Reverse Phase HPLC method for Simultaneous determination of active ingredients like Paracetamol, Caffeine and Codeine phosphate in pharmaceutical formulation. A mobile phase of Water:Acetonitrile:Methanol (60:15:25 v/v/v) was run on a C18 column, at the flow rate of 1 ml/min and UV detection was performed at 240 nm. The retention times were 9.13, 7.78 and 6.42 for Caffeine, Paracetamol and Codeine phosphate respectively. The RSD qualities are less then 2%, which showed that developed method was accurate and suitable for expected utilization. The method was validated with respect to the precision, accuracy and specificity of the Paracetamol, Caffeine and Codeine phosphate in pharmaceutical formulation.

CHAPTER - IV

AIMS AND OBJECTIVES

- Codeine phosphate and Triprolideine hydrochloride combination is an Antitussive, Analgesic and Antagonist of the H_1 histamine receptor. Codeine phosphate is often sold in combination with other ingredients such as in many cough analgesic medications.
- Codeine phosphate is an opioid analgesic which binds with stereo specific receptor at many sites within the CNS to alter processes affecting both the perception of pain and the emotional response. Triprolidine hydrochloride binds to the histamine H_1 receptor.
- Codeine phosphate and Triprolidine hydrochloride are official in the Indian Pharmacopoeia from the literature survey. It was found that there were only few RP-HPLC methods reported for the Simultaneous determination of content of Codeine phosphate and Triprolidine hydrochloride in cough syrup formulation.
- Hence, the main aims and objectives of the present work is to develop new RP-HPLC method for the Simultaneous determination of content of Codeine phosphate and Triprolidine hydrochloride in cough syrup formulation and to validate for the developed method by validated parameters (as per ICH guidelines) like System suitability, Accuracy, Precision, Specificity, Linearity, Robustness and Ruggedness.

CHAPTER - V

INSTRUMENTS AND CHEMICALS

Table No. 5. List of Instruments Used

S. No.	Name	Model
1	Weighing balance	BT 224 S / Sartorius
2	pH meter	pH 211 / Hanna
3	Sonicator	Ultrasonic Bath / PCI
4	HPLC – UV / PDA	1220 infinity / Agilent
5	Column	Inersustain / Inertsil

Table No. 6. List of Chemicals Used

S. No.	Name	Grade / Supplier
1	Methanol	HPLC / Moly Chemicals
2	Potassium dihydrogen orthophosphate	HPLC / Rankem Chemicals
3	Orthophosphoric acid	HPLC / Rankem Chemicals
4	Milli-Q water	HPLC / In House Production

Table No. 7. List of Active Pharmaceutical Ingredients Used

S. No.	Name	Specification
1	Codeine phosphate	Reference Standard
2	Triprolidine hydrochloride	Reference Standard

CHAPTER - VI

METHOD DEVELOPMENT

- The method development stage, decision regarding choice of column, mobile phase, detectors and method of quantitation must be addressed.
- In this way, development considers all the parameters pertaining to any methods. So here the trials mentioned describes how the optimization was done.

  - ***Selection of Stationary Phase:***

    Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drugs selected for the present study were polar compounds and could be separated either by Normal phase chromatography or Reverse phase chromatography. From literature survey, it was found that different C18 column could be appropriately used for the quantitation of Codeine phosphate and Triprolidine hydrochloride.

  - ***Selection of Mobile Phase:***

    The mobile phase was selected and chromatograms were recorded, trials were done on different mobile phase.

  - ***Selection of Wavelength:***

    The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substance and the drugs to be detected. The wavelength for measurement was selected as 280 nm from the absorption spectrum.

6.1 Reagent Preparation and Assay:

❖ **Preparation of Buffer Solution:**

8.1654 gm of potassium dihydrogen orthophosphate was weighed and dissolved in 1000 ml of water and pH was adjusted to 3.0 with orthophosphoric acid.

- **Solution A** - Buffer : Methanol (50:50)
- **Solution B** - Methanol : Buffer (50:50)
- **Mobile Phase** - Solution A : Solution B (55:45)
- **Diluents** - Mobile phase

❖ **Standard Preparation:**

Weigh accurately about 10 mg of Codeine phosphate and 12.5 mg Triprolidine hydrochloride working standards and transfer into a 50 ml volumetric flask, add 70 ml of diluents and sonicate to dissolve it completely and make the volume up to the mark with the same solvent. Further pipette 5 ml of the above stock solution into 25 ml volumetric flask and dilute up to the mark with diluents, mix well and filter through 0.45 µm filter. Inject 20 µl of the standard solution into a chromatographic system and measure the area for the Codeine phosphate and Triprolideine hydrochloride peaks.

❖ **Assay:**

Accurately pipette out 5 ml of the sample into a 100 ml volumetric flask and 70 ml of diluents was added and mixed well and made up to the mark with diluents. Mix well and filter through 0.45 µm filter. Measure the area for the Codeine phosphate and Triprolideine hydrochloride peaks and calculate the percentage assay by using formula.

❖ **Calculation:**

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Wt/ml}{Label\ claim} \times 100$$

Where,

AT = Peak area of sample solution

AS = Peak area of standard solution

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

6.2 Trials:

❖ **Trial:1**

➢ **Buffer Preparation:**

8.272 gm (0.08 M) potassium dihydrogen orthophosphate was weighed and transfered to 1000 ml standard flask and volume was made up with water and then pH was adjusted to 3.0 using orthophosphoric acid.

✓ **Solution A** - Buffer : Methanol (50:50)

✓ **Solution B** - Methanol : Buffer (50:50)

✓ **Mobile Phase** - Solution A : Solution B (55:45)

➢ **Chromatographic Conditions:**

❖ **Column** : Inersustain C18 (250 x 4.6 mm, 5µ)

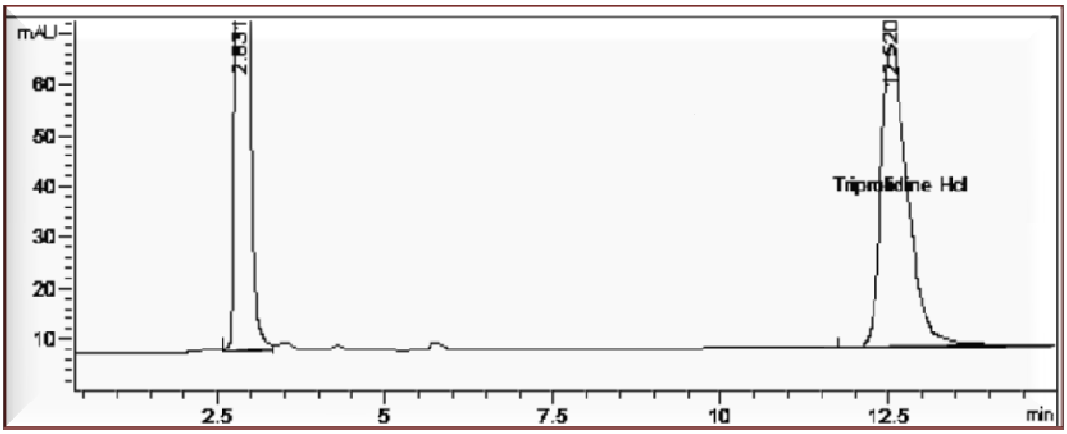
❖ **Detector Wavelength** : 210 nm

- ❖ **Column Temperature** : 30 ºC
- ❖ **Injection Volume** : 20 µl
- ❖ **Flow Rate** : 1.0 ml/min
- ❖ **Run Time** : 15 min

➢ **Observation:**

Peak shape was not good for Codeine phosphate and Triprolidine hydrochloride. The chromatogram was shown in **Figure No. 11**.

Figure No. 11. Chromatogram of Trial:1
(Codeine phosphate and Triprolidine hydrochloride)



❖ **Trial:2**

➢ **Buffer Preparation:**

8.0742 gm (0.04 M) potassium dihydrogen orthophosphate was weighed and transfered to 1000 ml standard flask and volume was made up with water and then pH was adjusted to 3.0 using orthophosphoric acid.

- ✓ **Solution A** - Buffer : Methanol (50:50)
- ✓ **Solution B** - Methanol : Buffer (50:50)
- ✓ **Mobile Phase** - Solution A : Solution B (55:45)

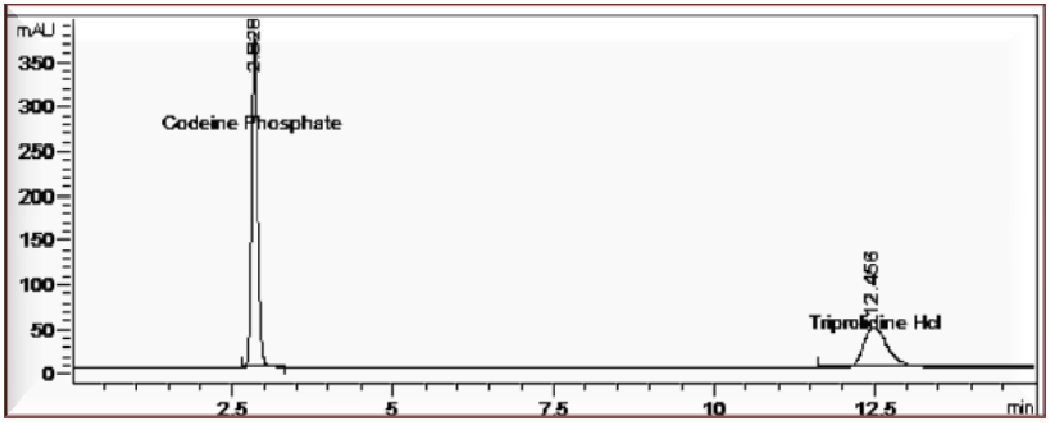
- **Chromatographic Conditions:**

❖ **Column**	**:**	Inersustain C18 (250 x 4.6 mm, 5µ)
❖ **Detector Wavelength**	**:**	240 nm
❖ **Column Temperature**	**:**	30 ºC
❖ **Injection Volume**	**:**	20 µl
❖ **Flow Rate**	**:**	1.0 ml/min
❖ **Run Time**	**:**	15 min

- **Observation:**

Peak shape was not good for Codeine phosphate and Triprolidine hydrochloride and also theoretical plate was less. The chromatogram was shown in **Figure No. 12**.

Figure No. 12. Chromatogram of Trial:2
(Codeine phosphate and Triprolidine hydrochloride)



❖ **Trial:3 (Optimized Method)**

- **Buffer Preparation:**

8.1654 gm (0.06 M) potassium dihydrogen orthophosphate was weighed and transfered to 1000 ml standard flask and volume was made up with water and then pH was adjusted to 3.0 using orthophosphoric acid.

- ✓ **Solution A** - Buffer : Methanol (50:50)
- ✓ **Solution B** - Methanol : Buffer (50:50)
- ✓ **Mobile Phase** - Solution A : Solution B (55:45)

➢ **Chromatographic Conditions:**

- ❖ **Column** : Inersustain C18 (250 x 4.6 mm, 5µ)
- ❖ **Detector Wavelength** : 280 nm
- ❖ **Column Temperature** : 30 ºC
- ❖ **Injection Volume** : 20 µl
- ❖ **Flow Rate** : 1.0 ml/min
- ❖ **Run Time** : 15 min

➢ **Observation:**

Resolution was satisfactory for Codeine phosphate and Triprolidine hydrochloride and also peak shape was good. The chromatogram was shown in **Figure No. 13** and the results obtained were tabulated as shown in **Table No. 8**.

Figure No. 13. Chromatogram of Trial:3 - Optimized Method (Codeine phosphate and Triprolidine hydrochloride)

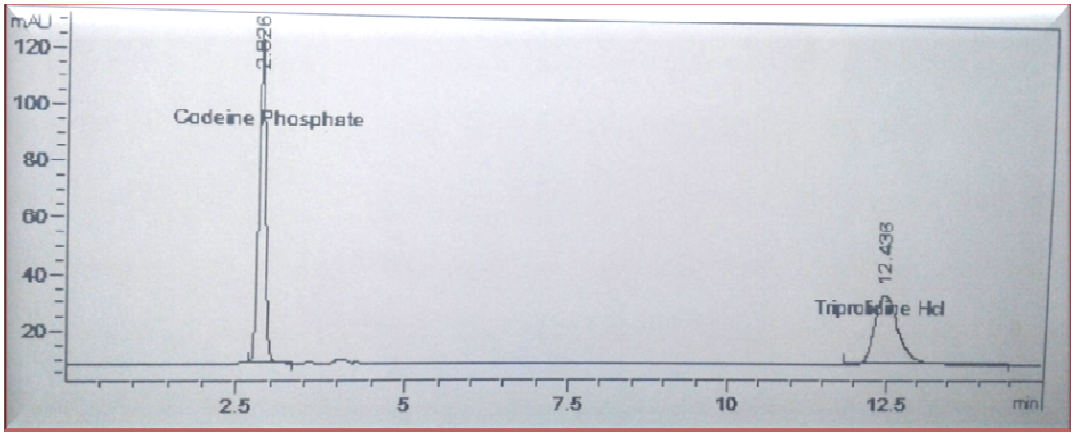


Table No. 8. Results of Trial:3 - Optimized Method

(Codeine phosphate and Triprolidine hydrochloride)

Name	RT	Area	Tailing Factor	Theoretical Plates	Resolution	Assay Value (%)
Codeine phosphate	2.826	812.508	0.74	3892	0.00	99.5
Triprolidine hydrochloride	12.436	696.607	0.59	5182	22.0	99.9

CHAPTER - VII

METHOD VALIDATION

7.1 Validation:

- According to the ICH guidelines, Method Validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specification and quality, reliability and consistency of analytical results. It is an integral part of any good analytical practice.
- Such validation analytical method for qualitative and quantitative testing of the drug molecule assume greater importance when they are employed to generate quality and safety compliance data during development, pre-formulation studies and post approval of drug products.

7.2 Validation Parameters:

- Typical analytical parameters used in assay validation include,
  - **A)** System Suitability
  - **B)** Accuracy
  - **C)** Precision
  - **D)** Specificity
  - **E)** Linearity
  - **F)** Robustness
  - **G)** Ruggedness

A) System Suitability:

- System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analytical constitute an integral system that can be evaluated as such.
- System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated and the parameters like retention time, area, tailing factor and theoretical plates are determined and the chromatogram for the system suitability is shown in **Figure No. 14** and the results obtained were tabulated as shown in **Table No. 9** to **10**.

★ **Acceptance Criteria:**

- % RSD of peak area should NMT 2.0.
- The tailing factor for Codeine phosphate and Triprolidine hydrochloride peak should NMT 2.0.
- The column efficiency for Codeine phosphate and Triprolidine hydrochloride should NLT 3000 theoretical plates.

B) Accuracy:

- The accuracy of an analytical procedure express the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.
- Accuracy should be reported as percent recovery by the assay of known added amount of analytes in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Accuracy may be inferred once precision, linearity and specificity have been established.

★ **Procedure:**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).

❖ **Preparation of 50% Sample Solution:**

- Accurately weigh and transfer 100 µg of Codeine phosphate and 15 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).

❖ **Preparation of 100% Sample Solution:**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).

❖ **Preparation of 150% Sample Solution:**

- Accurately weigh and transfer 300 µg of Codeine phosphate and 30 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask add about 70 ml of diluents and sonicate to dissolve it completely and volume up to mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).

Inject 20 µl of placebo and standard solution of accuracy-50%, accuracy-100% and accuracy-150% solutions into HPLC. Now calculate the amount obtained and amount added for Codeine phosphate and Triprolidine hydrochloride samples. Calculate the concentration in µg/ml in the spiked placebo in all the above cases by comparing with the standard solution. Calculate the individual recovery and mean recovery values. The chromatogram for the accuracy is shown in **Figure No. 15** to **17** and the results obtained were tabulated (including overall Mean, SD and % RSD) as shown in **Table No. 11** to **14.** Also % recovery study results for Codeine phosphate and Triprolidine hydrochloride are shown in **Table No. 15**.

★ **Calculation:**

$$\textbf{\% Recovery} = \frac{\text{Amount recovered}}{\text{Actual amount added}} \times 100$$

★ **Acceptance Criteria:**

% recovery in all the cases should be between 100±2 %.

C) Precision:

- The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample.
- Precision may be considered at three levels**:** repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples using a minimum of 6 determinations at 100 percent of the test concentration. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

★ **Procedure:**

i) *System Precision*:

Six replicate injections of standard solution were injected into the HPLC system.

❖ **Preparation of Standard Solution (100 µg/ml):**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).
- Inject 20 µl of the blank solution and six replicate injections of standard solution of 100 µg/ml and calculate the % RSD for the area of six replicate injections. The chromatogram for system precision including blank is

shown in **Figure No.18** to **19** and the results obtained were tabulated as shown in **Table No. 16**.

ii) *Method Precision*:

Six replicate injections of sample solution were injected into the HPLC system.

❖ **Preparation of Sample Solution (100 µg/ml):**

- Accurately pipette out 5 ml of the sample into a 100 ml volumetric flask and 70 ml of diluents was added and mixed well and make volume up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).
- Inject 20 µl of the blank solution and six replicate injections of sample solution of 100 µg/ml and calculate the % RSD for the area of six replicate injections. The chromatogram for method precision including blank is shown in **Figure No. 18** and **Figure No. 20** to **25** and the results obtained were tabulated (including overall Mean, SD and % RSD) as shown in **Table No. 17** to **23**.

★ **Calculation:**

$$\% \text{ RSD} = (\sigma/\mu)*100$$

★ **Acceptance Criteria:**

- % RSD for the area of Codeine phosphate and Triprolidine hydrochloride from the sample chromatogram should NMT 1.0.

D) Specificity:

➢ The specificity of the method corresponds to the non-interaction of the placebo with that of the active particle. Typically these might include impurities, degradants, matrix etc.

★ **Procedure:**

❖ **Preparation of Standard Solution:**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
- Further pipette 5ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter). Inject 20 µl of blank solution into the HPLC system and observe chromatogram.
- Inject 20 µl of standard solution for six times into the HPLC system and compare the chromatogram visually and check for any interference. Calculate the % RSD for the area of six replicate injections. The chromatogram for specificity including blank is shown in **Figure No. 26** and **Figure No. 27** to **32** and the results obtained were tabulated (including overall Mean, SD and % RSD) as shown in **Table No. 24** to **30.**

★ **Calculation:**

$$\% \text{ RSD} = (\sigma/\mu)*100$$

★ **Acceptance Criteria:**

- There should not be any peak in the blank and placebo solution run at the retention time corresponding to Codeine phosphate and Triprolidine hydrochloride as in standard run.

E) Linearity:

- The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The procedure used for linearity was carried out as per ICH Q2 (R1) guidelines.
- A linear relationship should be evaluated across the range of the analytical procedure by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculated of correlation coefficient, y-intercept, slope of the regression line and residual sum of squares. A plot of the data should be included. For the establishment of linearity, a minimum of five concentrations is recommended.

★ **Procedure:**

❖ **Preparation of Standard Solution:**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

❖ **Preparation of Sample Solution:**

- From the above stock solution, pipette out 5.0, 7.5, 10.0, 12.5 and 15.0 ml respectively into individual 50 ml of volumetric flasks and dilute up to the mark with diluents to prepare 50, 75, 100, 125 and 150 µg/ml of sample solution respectively. Mix well and filter through 0.22 µm filter (Membrane filter).

- Inject 20 µl of blank solution and standard solutions into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on x-axis concentration and on y-axis peak area) and calculate the correlation coefficient. The chromatogram for linearity including overlay is shown in **Figure No. 33** to **40** and the results obtained were tabulated as shown in **Table No. 31** to **37.**

★ **Calculation:**

$$Correl(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

Regression line (y) = mx+c

(Where, **m** = slope, **c** = y-intercept)

★ **Acceptance Criteria:**

- Correlation coefficient should NLT 0.995.

F) Robustness:

- The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The procedure used for robustness was carried out as per ICH Q2 (R1) Guidelines.
- To perform the robustness of the method, deliberate change the conditions like flow rate as well as pH of buffer solution in mobile phase and to evaluate the impact on the method.

★ **Procedure:**

❖ **Preparation of Standard Solution (100 µg/ml):**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml of volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).
- Inject 20 µl of the blank solution and the standard solution of 100 µg/ml for five times and analyzed using varied flow rates (1.2 ml and 1.4 ml) along with optimized method flow rate and the pH of buffer solution in mobile phase was varied at +/- 0.1 from the normal pH of 2.3. Finally calculate the % RSD for the area of five replicate injections. The chromatogram for robustness including blank is shown in **Figure No. 41** and **Figure No. 42** to **53** and the results obtained were tabulated (including overall Mean, SD and % RSD) as shown in **Table No. 38** to **50**.

★ **Calculation:**

$$\% \text{ RSD} = (\sigma/\mu)*100$$

★ **Acceptance Criteria:**

- The effect of change in the flow rate and pH of buffer solution in mobile phase, % RSD for the area of Codeine phosphate and Triprolidine hydrochloride should NMT 1.0.

G) Ruggedness:

- Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst).
- Ruggedness of the current method was demonstrated by analyzing two samples of syrup formulation by two different analysts in the same laboratory.

★ **Procedure:**

❖ **Preparation of Standard Solution (100 µg/ml):**

- Accurately weigh and transfer 200 µg of Codeine phosphate and 25 µg of Triprolidine hydrochloride working standards into a 100 ml volumetric flask, add about 70 ml of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
- Further pipette 5 ml of the above stock solution into a 25 ml of volumetric flask and dilute up to the mark with diluents. Mix well and filter through 0.22 µm filter (Membrane filter).
- Inject 20 µl of the blank solution and the standard solution of 100 µg/ml for six times and analyzed by two different analysts in the same laboratory. Finally calculate the % RSD for the area of twelve replicate injections. The chromatogram for ruggedness including blank is shown in **Figure No. 54** and **Figure No. 55** to **56** and the results obtained were tabulated (including overall Mean, SD and % RSD) as shown in **Table No. 51** to **54.**

★ **Calculation:**

$$\% \text{ RSD} = (\sigma/\mu)*100$$

★ **Acceptance Criteria:**

- % RSD for the area of Codeine phosphate and Triprolidine hydrochloride should NMT 1.0.

CHAPTER - VIII

RESULTS AND DISCUSSION

8.1 System Suitability:

Figure No. 14. Chromatogram of System Suitability

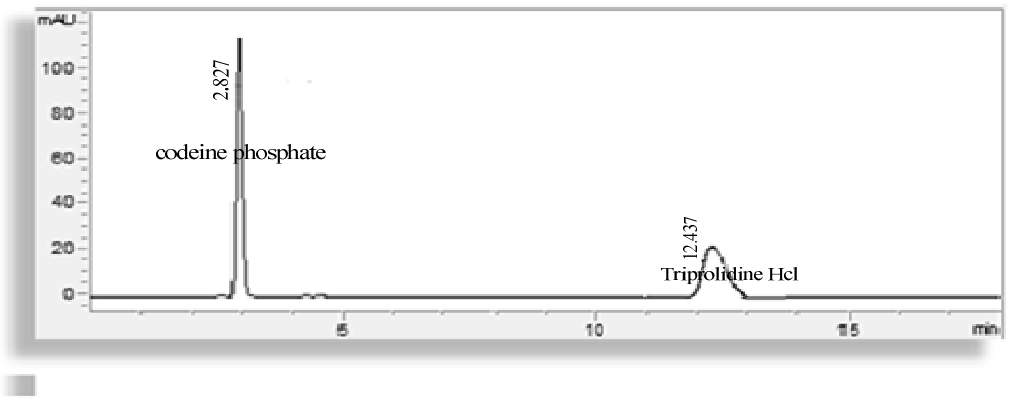


Table No. 9. Results of System Suitability (Codeine phosphate)

No. of Injection	RT	Area	Tailing Factor	Theoretical Plates
1	2.827	825335	0.74	3892
2	2.823	825338	0.73	3795
3	2.825	825330	0.73	3852
4	2.822	825337	0.75	3845
5	2.826	825333	0.72	3862
6	2.828	824388	0.71	3895
Mean	2.8251	825176.8	0.73	3856.833
SD	0.0023	386.4585	0.014	36.62467
% RSD	0.091	0.04	1.9	0.9496

Table No. 10. Results of System Suitability (Triprolidine hydrochloride)

No. of Injection	RT	Area	Tailing Factor	Theoretical Plates
1	12.437	696605	0.66	5185
2	12.432	696573	0.65	5179
3	12.435	695605	0.67	5183
4	12.433	695793	0.64	5181
5	12.438	694599	0.67	5178
6	12.436	696703	0.65	5182
Mean	12.435	695988	0.656	5186
SD	0.0023	755.2234	0.0121	5.5136
% RSD	0.018	0.118	1.8	0.1

8.2 Accuracy:

Figure No. 15. Chromatogram of Accuracy (50% Spike Solution)

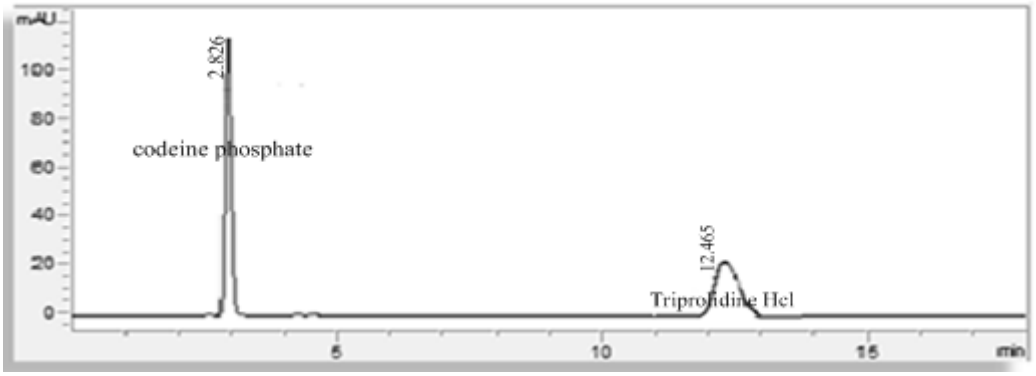


Table No. 11. Results of Accuracy (50% Spike Solution)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.826	865929	0.73	4817
Triprolidine hydrochloride	12.485	694297	0.61	5184

Figure No. 16. Chromatogram of Accuracy (100% Spike Solution)

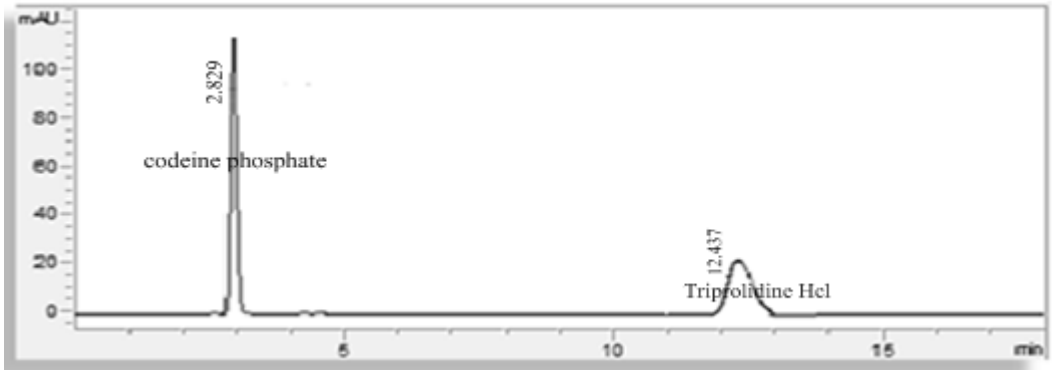


Table No. 12. Results of Accuracy (100% Spike Solution)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.829	865934	0.73	4320
Triprolidine hydrochloride	12.433	694296	0.61	5181

Figure No. 17. Chromatogram of Accuracy (150% Spike Solution)

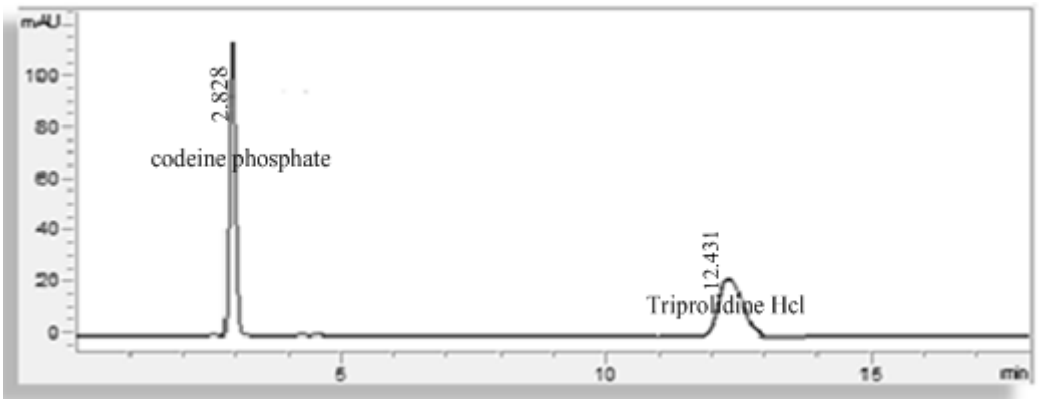


Table No. 13. Results of Accuracy (150% Spike Solution)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.828	865933	0.74	4314
Triprolidine hydrochloride	12.431	694294	0.60	5185

Table No. 14. Results of Accuracy (Overall Mean, SD, % RSD for Codeine phosphate and Triprolidine hydrochloride)

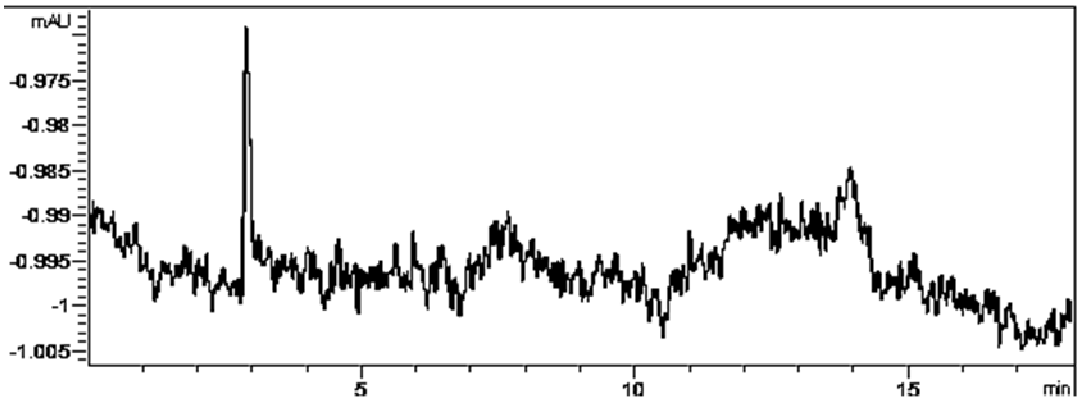
Name		RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	**Mean**	2.8486	865932	0.74	3873.17
	SD	0.0025	3.7416	0.008	41.5953
	% RSD	0.8	0.0004	1.081	1.0
Triprolidine hydrochloride	**Mean**	13.6585	694295.6	0.6216	4904
	SD	0.00207	2.16	0.0116	4. 979
	% RSD	0.014	0.0002	1.8	0.1

Table No. 15. Results of % Recovery for Codeine phosphate and Triprolidine hydrochloride

Injection Sample	Spike Level (%)	Amount Present (µg)	Amount Recovered (µg)	% Recovered	Mean Recovery (%)	Accep. Criteria (%)
Codeine phosphate	50	100	99.8	99.8	99.8	100 ± 2.0
	100	200	199.8	99.9		
	150	300	300.07	99.8		
Triprolidine HCl	50	15	15	100.0	100.0	100 ± 2.0
	100	25	25	100.0		
	150	35	35	100.0		

8.3 Precision:

Figure No. 18. Chromatogram of Precision (Blank)



i) System Precision:

Figure No. 19. Chromatogram of System Precision (Standard)

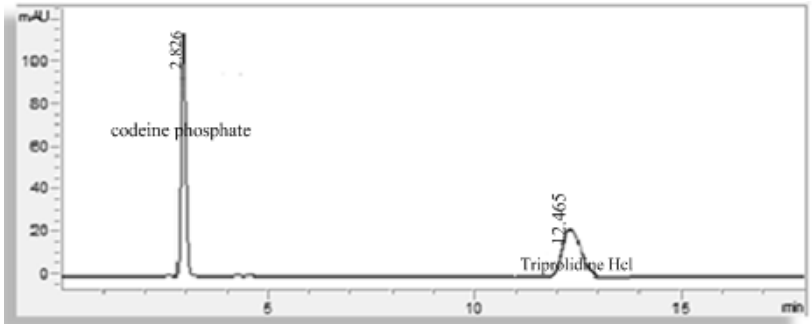


Table No. 16. Results of System Precision (Standard)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.826	865929	0.73	4317
Triprolidine hydrochloride	12.465	694298	0.60	5184

ii) Method Precision:

Figure No. 20. Chromatogram of Method Precision (Sample - 1)

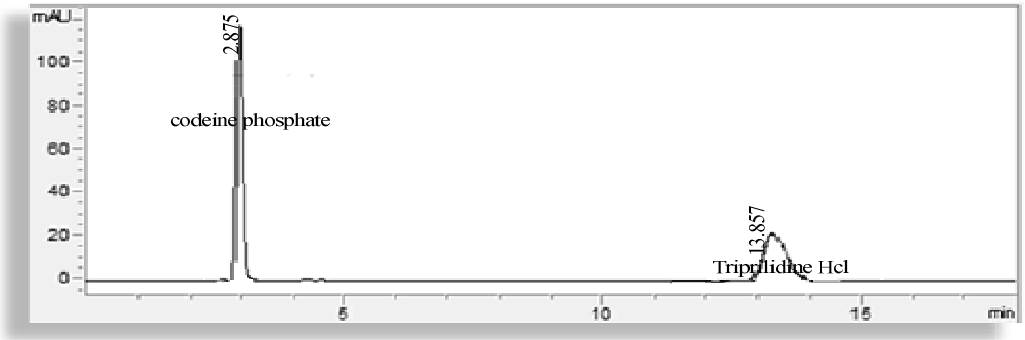


Table No. 17. Results of Method Precision (Sample - 1)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.875	865909	0.73	3858
Triprolidine hydrochloride	13.857	694289	0.62	4898

Figure No. 21. Chromatogram of Method Precision (Sample - 2)

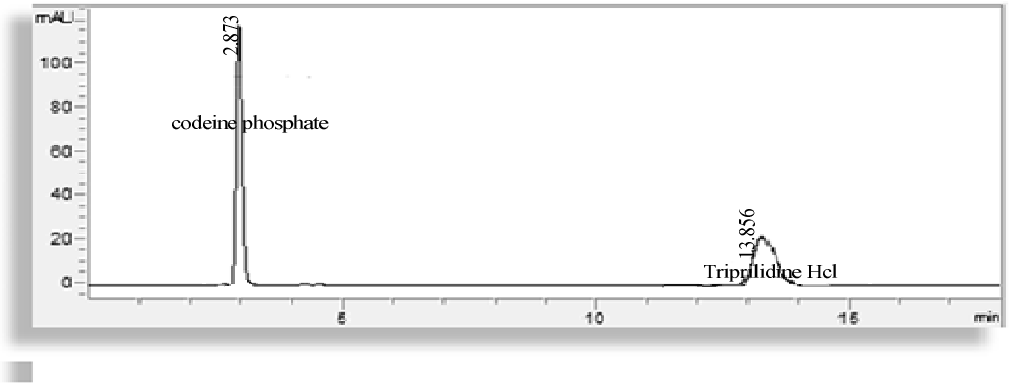


Table No. 18. Results of Method Precision (Sample - 2)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.873	865912	0.75	3857
Triprolidine hydrochloride	13.856	694292	0.63	4894

Figure No. 22. Chromatogram of Method Precision (Sample - 3)

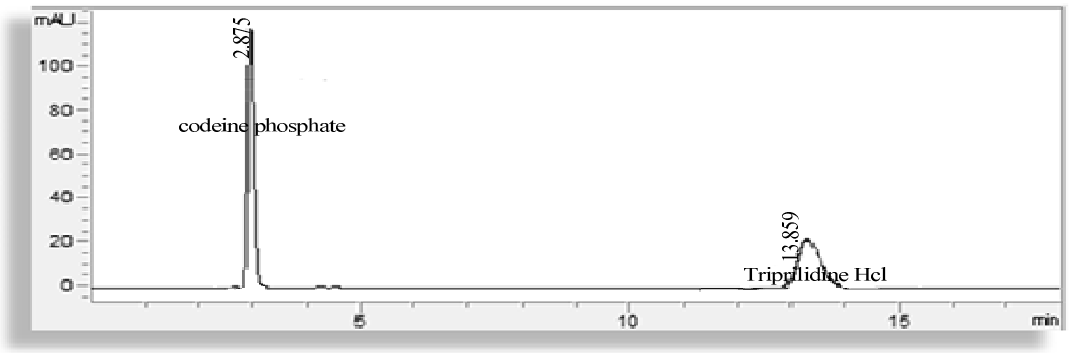


Table No. 19. Results of Method Precision (Sample - 3)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.875	865918	0.73	3056
Triprolidine hydrochloride	13.859	694295	0.62	4893

Figure No. 23. Chromatogram of Method Precision (Sample - 4)

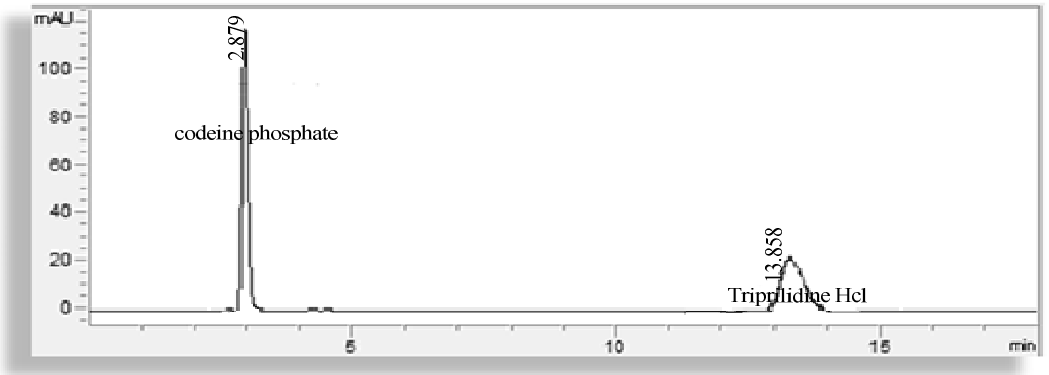


Table No. 20. Results of Method Precision (Sample - 4)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.879	865911	0.74	3859
Triprolidine hydrochloride	13.858	694293	0.64	4899

Figure No. 24. Chromatogram of Method Precision (Sample - 5)

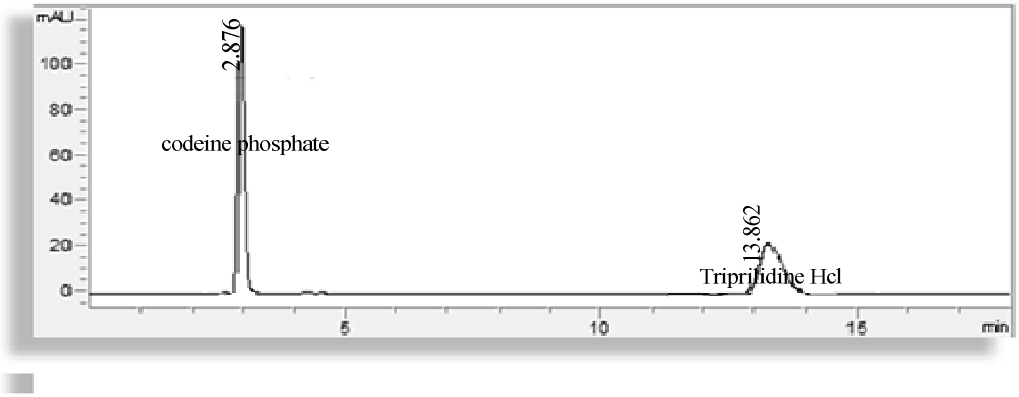


Table No. 21. Results of Method Precision (Sample - 5)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.876	865908	0.75	3855
Triprolidine hydrochloride	13.862	694296	0.61	4896

Figure No. 25. Chromatogram of Method Precision (Sample - 6)

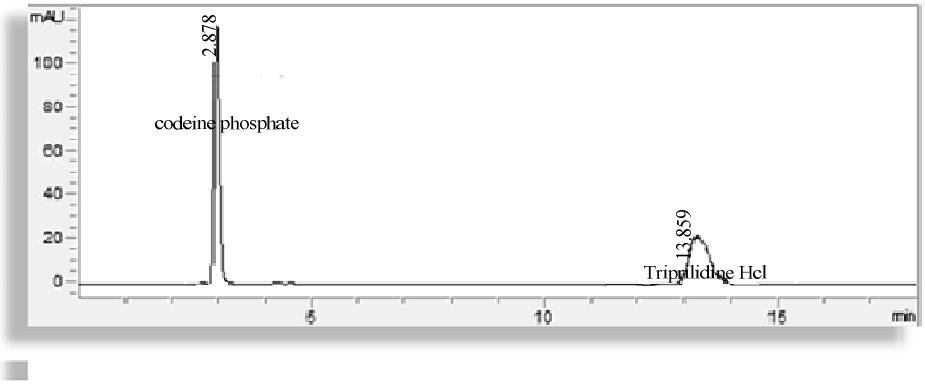


Table No. 22. Results of Method Precision (Sample - 6)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.878	865919	0.73	3854
Triprolidine hydrochloride	13.859	694294	0.61	4897

Table No. 23. Results of Method Precision (Overall Mean, SD, % RSD for Codeine phosphate and Triprolidine hydrochloride)

Name		RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	**Mean**	2.8486	865935	0.74	3873.17
	SD	0.0025	2.984	0.008	41.5953
	% RSD	0.8	0.00034	1.081	0.1
Triprolidine hydrochloride	**Mean**	13.6585	694291	0.6216	4904
	SD	0.00207	2.5819	0.0116	4. 979
	% RSD	0.014	0.00037	1.8	0.1

8.4 Specificity:

Figure No. 26. Chromatogram of Specificity (Blank)

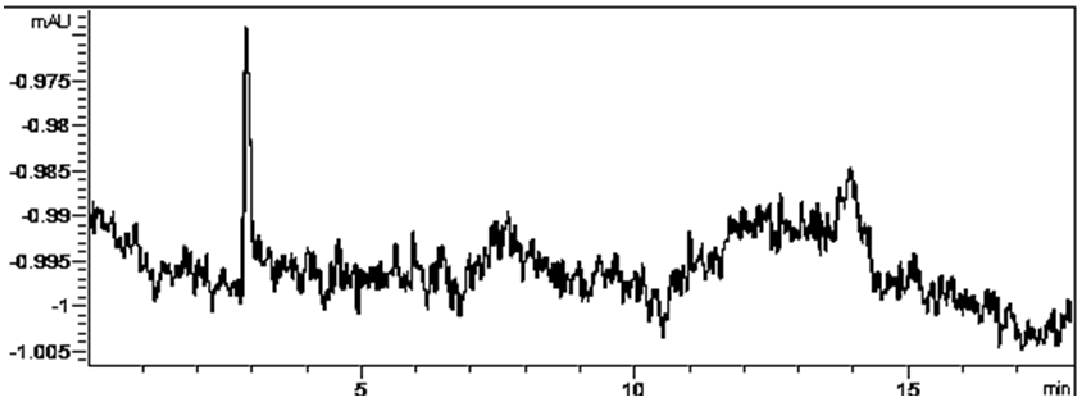


Figure No. 27. Chromatogram of Specificity (Standard - 1)

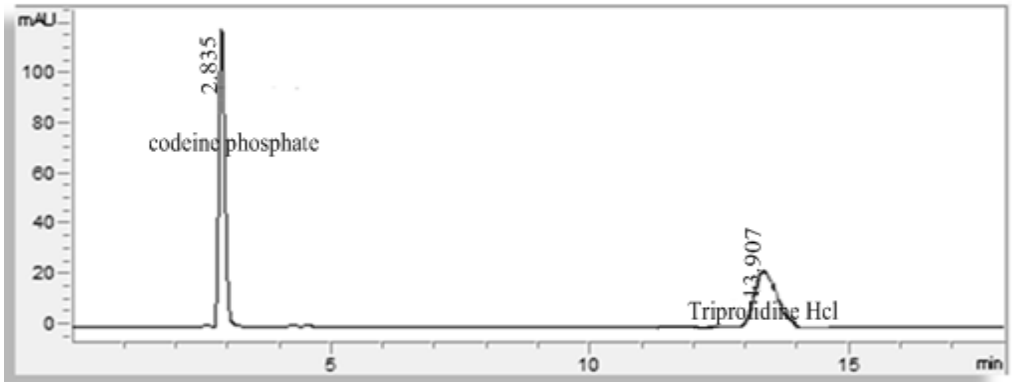


Table No. 24. Results of Specificity (Standard - 1)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.835	812508	0.71	3782
Triprolidine hydrochloride	13.907	696610	0.60	4867

Figure No. 28. Chromatogram of Specificity (Standard - 2)

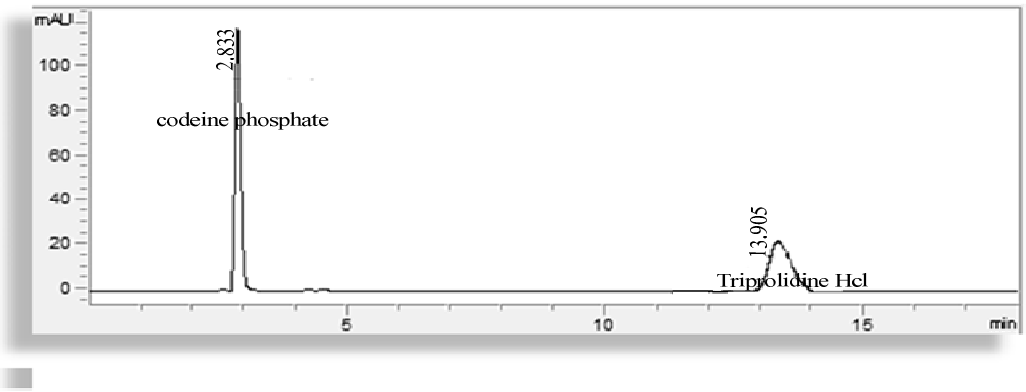


Table No. 25. Results of Specificity (Standard - 2)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.833	812619	0.72	3785
Triprolidine hydrochloride	13.905	696605	0.61	4863

Figure No. 29. Chromatogram of Specificity (Standard - 3)

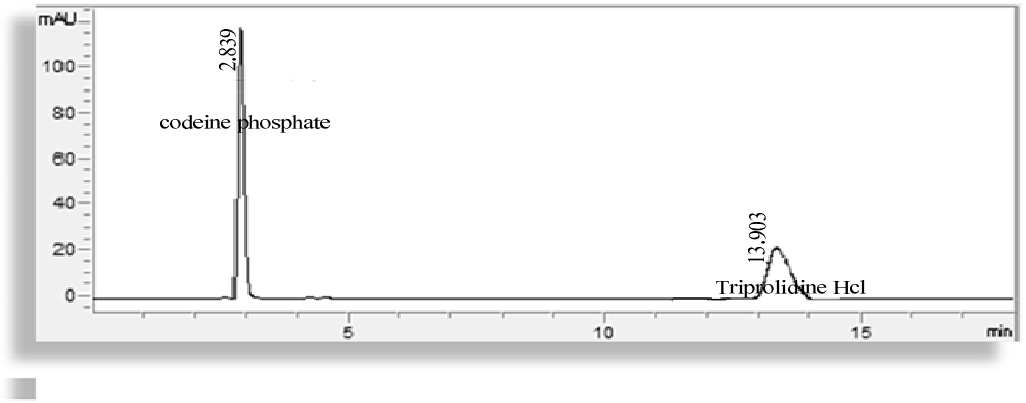


Table No. 26. Results of Specificity (Standard - 3)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.839	812512	0.70	3784
Triprolidine hydrochloride	13.903	696612	0.61	4865

Figure No. 30. Chromatogram of Specificity (Standard - 4)

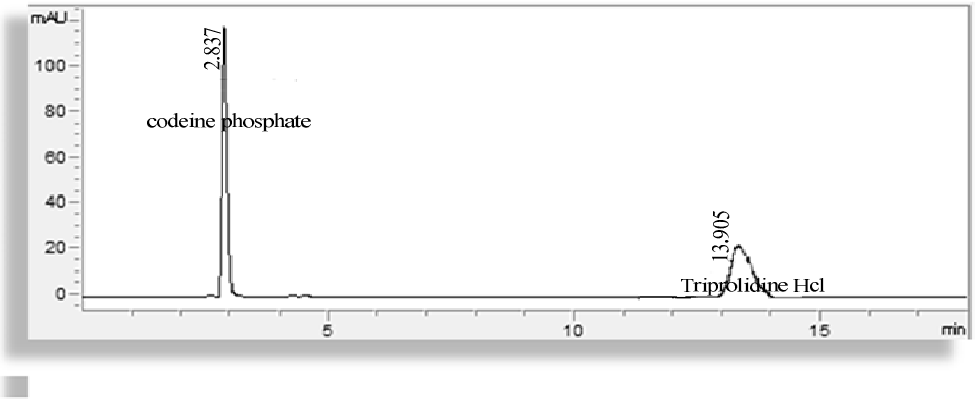


Table No. 27. Results of Specificity (Standard - 4)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.837	812613	0.71	3783
Triprolidine hydrochloride	13.905	696612	0.60	4862

Figure No. 31. Chromatogram of Specificity (Standard - 5)

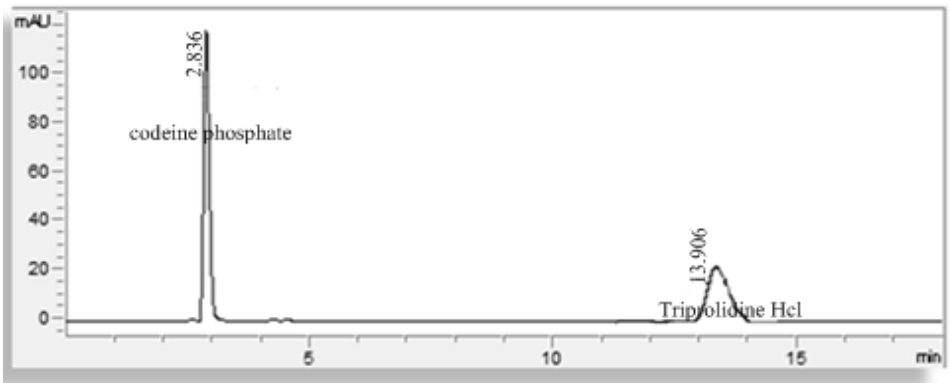


Table No. 28. Results of Specificity (Standard - 5)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.836	812509	0.70	3782
Triprolidine hydrochloride	13.906	696609	0.61	4863

Figure No. 32. Chromatogram of Specificity (Standard - 6)

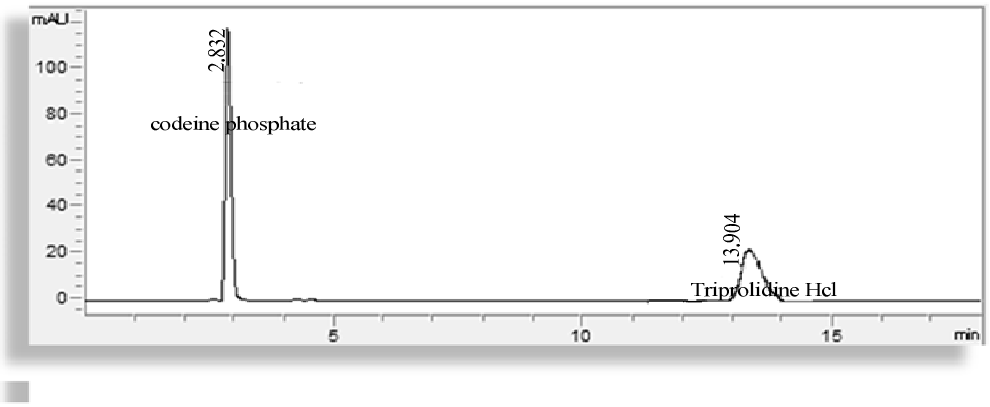


Table No. 29. Results of Specificity (Standard - 6)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.832	812505	0.71	3781
Triprolidine hydrochloride	13.904	696603	0.60	4860

Table No. 30. Results of Specificity (Overall Mean, SD, % RSD for Codeine phosphate and Triprolidine hydrochloride)

Name		RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	**Mean**	2.8353	812544.3	0.70833	3782.833
	SD	0.0025	55.59027	0.00752	1.471
	% RSD	0.088	0.006	1.0	0.03
Triprolidine hydrochloride	**Mean**	13.905	696607.8	0.605	4863
	SD	0.0014	3.3115	0.005477	1.4142
	% RSD	0.01	0.0004	0.90	0.02

8.5 Linearity:

Figure No. 33. Chromatogram of Linearity - 1 (50 µg/ml)

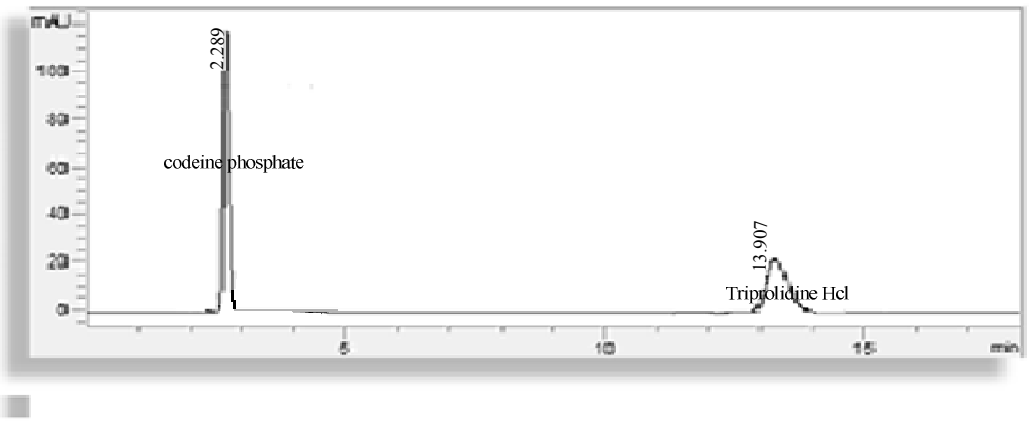


Table No. 31. Results of Linearity - 1 (50 µg/ml)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.289	294820	0.73	3793
Triprolidine hydrochloride	13.907	524063	0.61	4851

Figure No. 34. Chromatogram of Linearity - 2 (75 µg/ml)

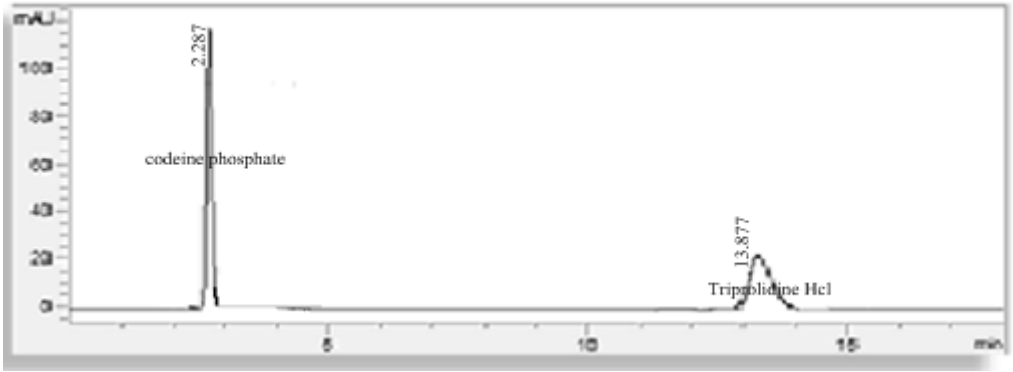


Table No. 32. Results of Linearity - 2 (75 µg/ml)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.287	446999	0.72	3858
Triprolidine hydrochloride	13.877	792940	0.61	4898

Figure No. 35. Chromatogram of Linearity - 3 (100 µg/ml)

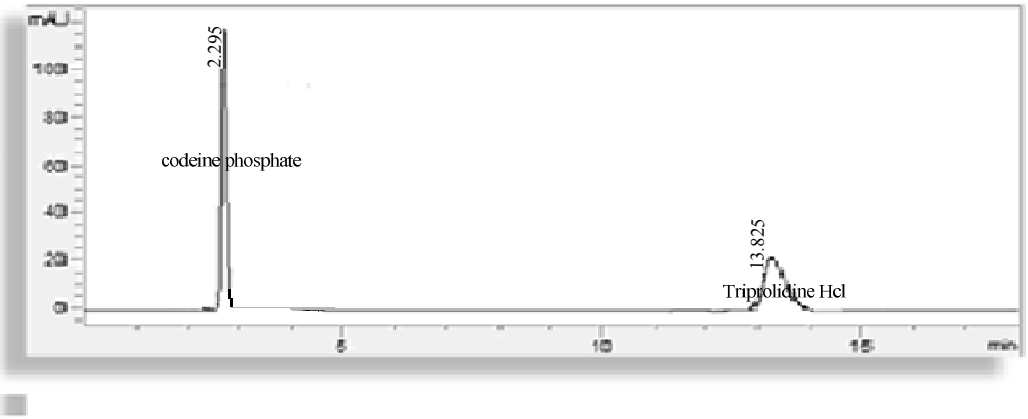


Table No. 33. Results of Linearity - 3 (100 µg/ml)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.295	595115	0.73	3851
Triprolidine hydrochloride	13.825	1063971	0.60	5004

Figure No. 36. Chromatogram of Linearity - 4 (125 µg/ml)

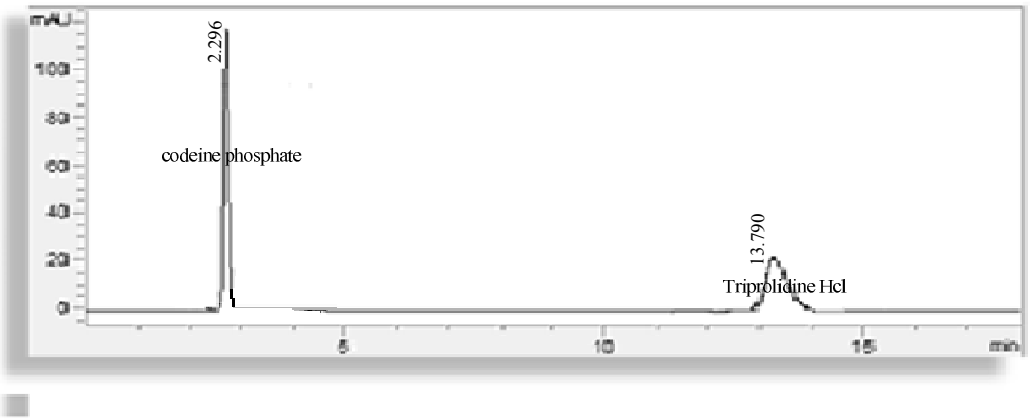


Table No. 34. Results of Linearity - 4 (125 µg/ml)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.296	748334	0.74	3798
Triprolidine hydrochloride	13.790	1342834	0.60	4906

Figure No. 37. Chromatogram of Linearity - 5 (150 µg/ml)

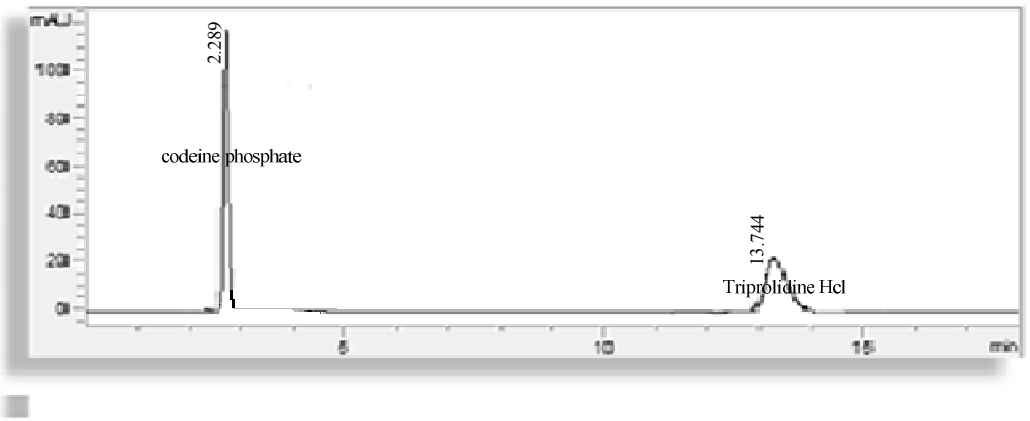


Table No. 35. Results of Linearity - 5 (150 µg/ml)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.289	898406	0.74	3770
Triprolidine hydrochloride	13.744	1618163	0.60	4874

Figure No. 38. Chromatogram of Overlay of Linearity 1 - 5 (50 to 150 µg/ml)

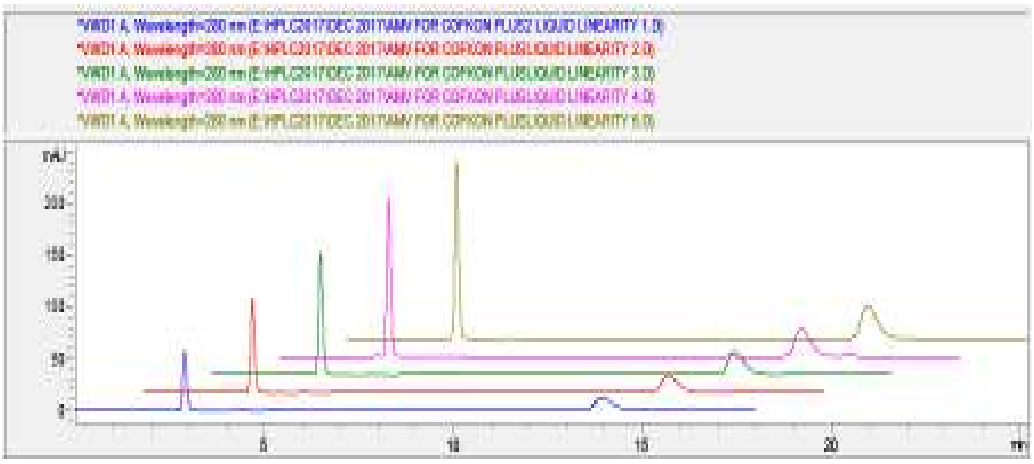


Figure No. 39. Chromatogram of Linearity Level 1 - 5 (50 to 150 µg/ml) for Codeine phosphate

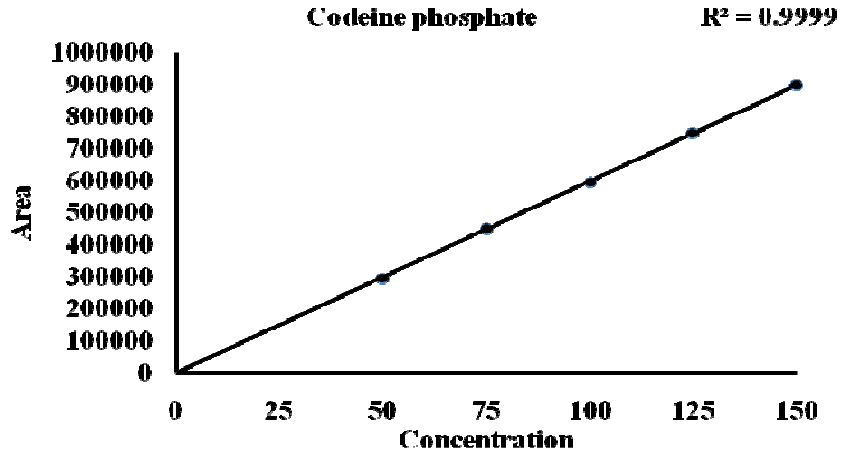


Table No. 36. Results of Linearity Level 1 - 5 (50 to 150 µg/ml) for Codeine phosphate

Linearity level	Concentration (µg/ml)	Area
1	50	294820
2	75	446999
3	100	595115
4	125	748334
5	150	898406
Correlation Coefficient		**0.9999901**

Figure No. 40. Chromatogram of Linearity Level 1 - 5 (50 to 150 µg/ml) for Triprolidne hydrochloride

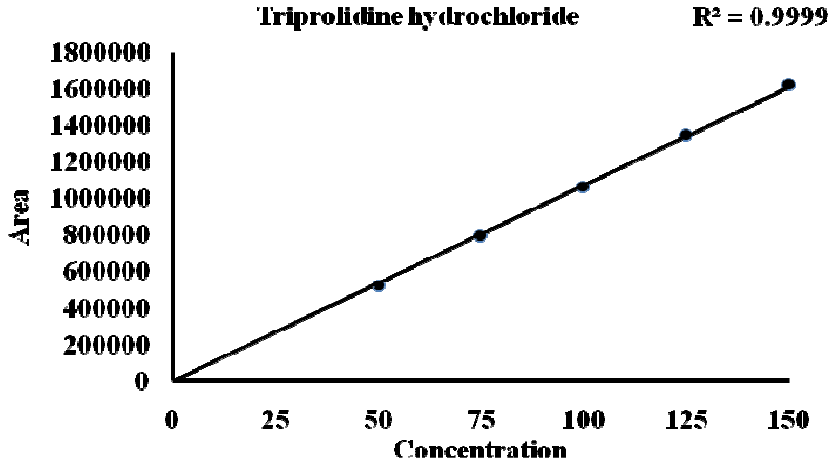
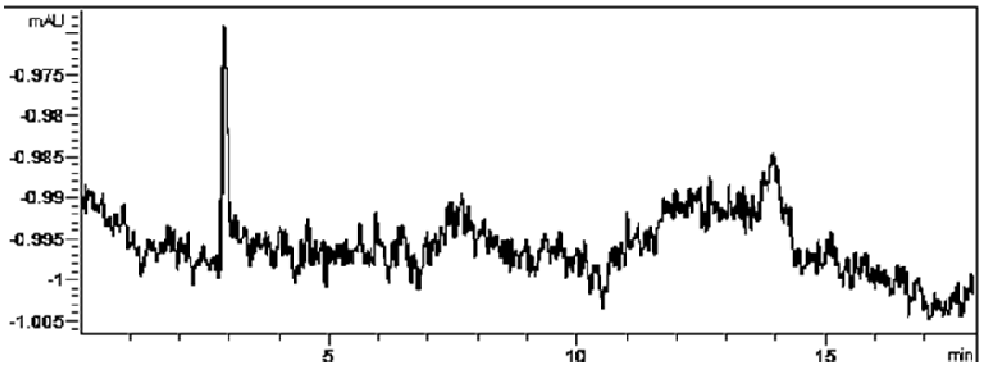


Table No. 37. Results of Linearity Level 1 - 5 (50 to 150 µg/ml) for Triprolidne hydrochloride

Linearity level	Concentration (µg/ml)	Area
1	50	524063
2	75	792940
3	100	1063971
4	125	1342834
5	150	1618163
Correlation Coefficient		**0.999974**

8.6 Robustness:

Figure No. 41. Chromatogram of Robustness (Blank)



i) Flow Rate Variation - 1 (1.2 ml/min):

Figure No. 42. Chromatogram of Robustness - Standard Solution 1 (Flow Rate - 1.2 ml/min)

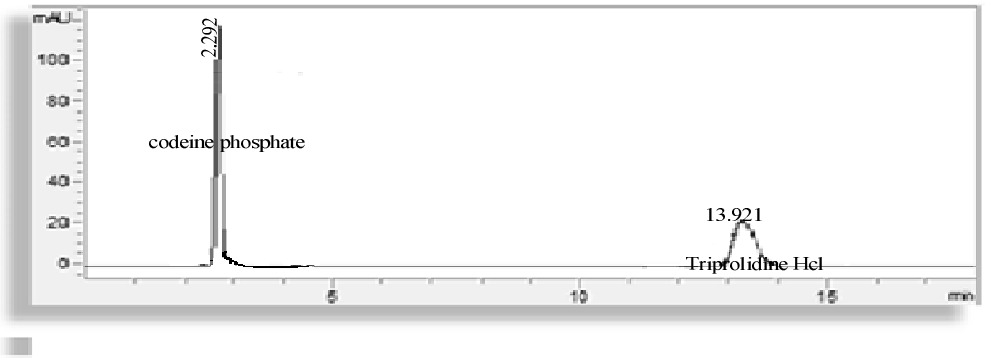


Table No. 38. Results of Robustness - Standard Solution 1 (Flow Rate - 1.2 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.292	920909	0.74	3757
Triprolidine hydrochloride	13.921	614097	0.63	4862

Figure No. 43. Chromatogram of Robustness - Standard Solution 2 (Flow Rate - 1.2 ml/min)

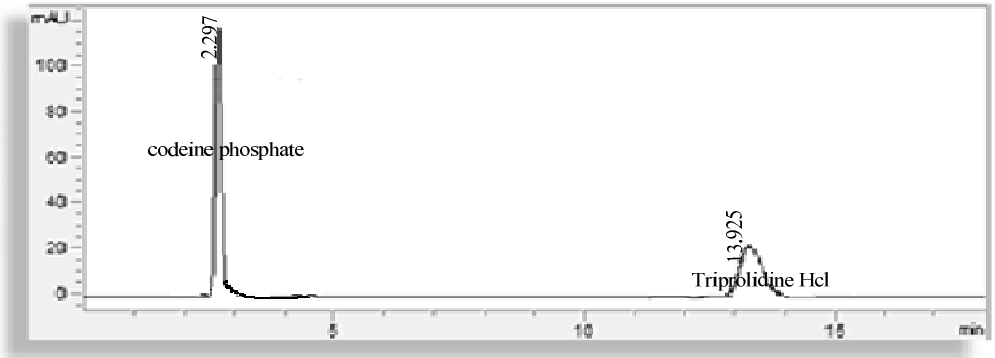


Table No. 39. Results of Robustness - Standard Solution 2

(Flow Rate - 1.2 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.297	920905	0.72	3752
Triprolidine hydrochloride	13.925	617095	0.67	4865

Figure No. 44. Chromatogram of Robustness - Standard Solution 3

(Flow Rate - 1.2 ml/min)

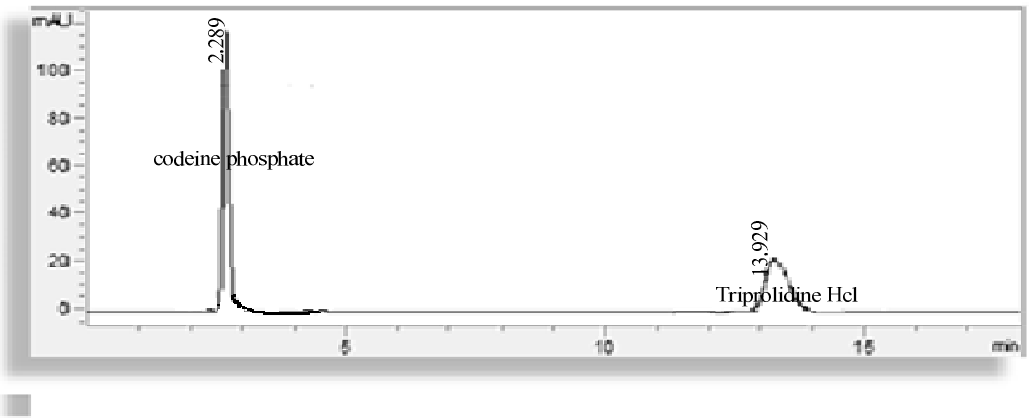


Table No. 40. Results of Robustness - Standard Solution 3

(Flow Rate - 1.2 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.289	920909	0.71	3715
Triprolidine hydrochloride	13.929	614093	0.63	4869

Figure No. 45. Chromatogram of Robustness - Standard Solution 4 (Flow Rate - 1.2 ml/min)

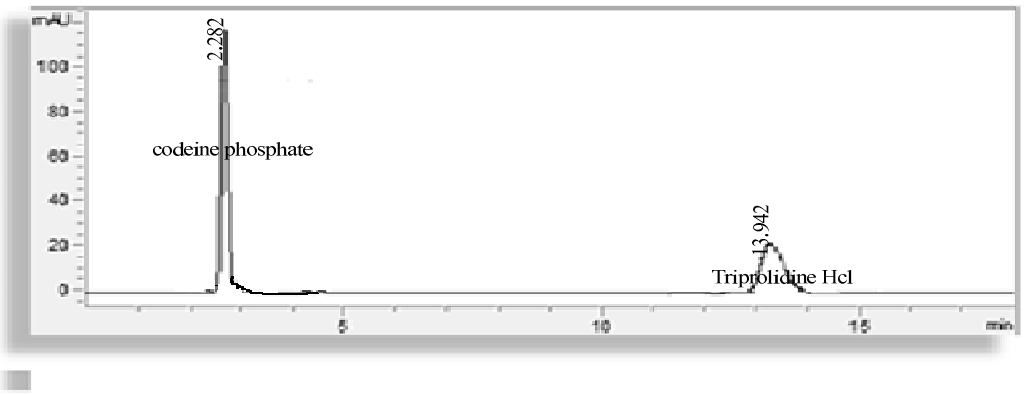


Table No. 41. Results of Robustness - Standard Solution 4 (Flow Rate - 1.2 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.282	920907	0.73	3719
Triprolidine hydrochloride	13.942	615099	0.64	4867

Figure No. 46. Chromatogram of Robustness - Standard Solution 5 (Flow Rate - 1.2 ml/min)

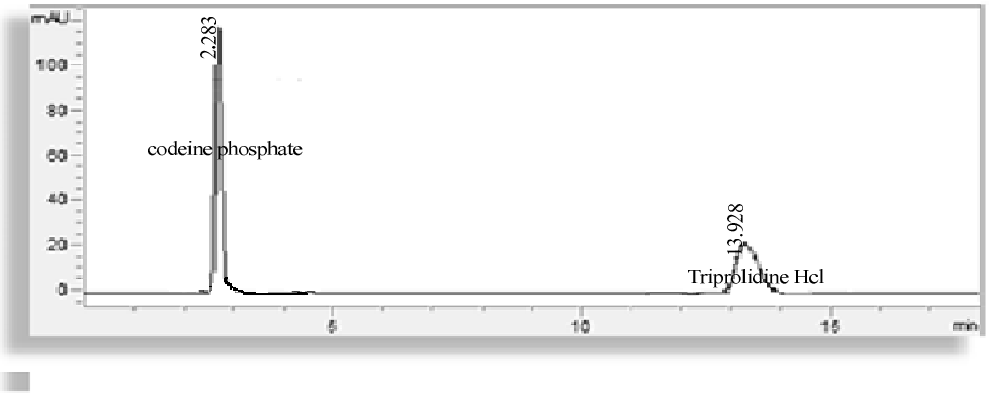


Table No. 42. Results of Robustness - Standard Solution 5

(Flow Rate - 1.2 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.283	9250904	0.73	3758
Triprolidine hydrochloride	13.928	614096	0.64	4862

ii) Flow Rate Variation - 2 (1.4 ml/min):

Figure No. 47. Chromatogram of Robustness - Standard Solution 1

(Flow Rate - 1.4 ml/min)

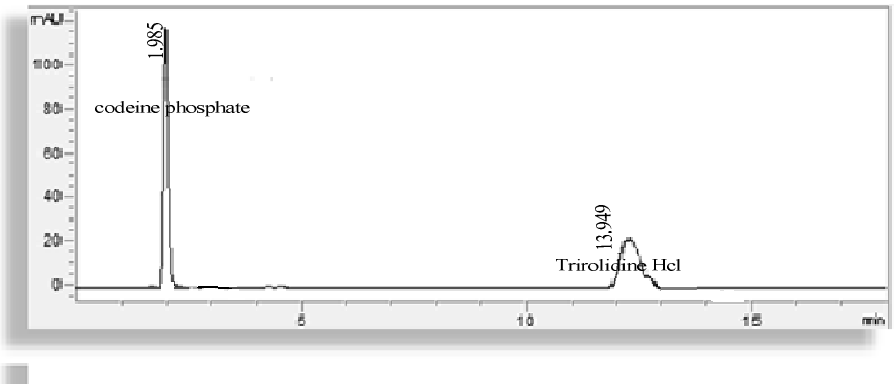


Table No. 43. Results of Robustness - Standard Solution 1

(Flow Rate - 1.4 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	1.985	829932	0.73	3894
Triprolidine hydrochloride	13.949	624587	0.60	4865

Figure No. 48. Chromatogram of Robustness - Standard Solution 2 (Flow Rate - 1.4 ml/min)

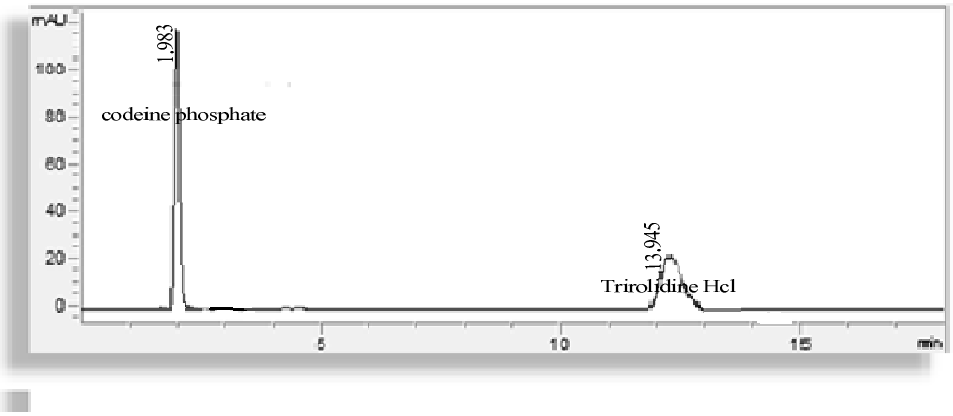


Table No. 44. Results of Robustness - Standard Solution 2 (Flow Rate - 1.4 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	1.983	825735	0.74	3899
Triprolidine hydrochloride	13.945	624289	0.61	4877

Figure No. 49. Chromatogram of Robustness - Standard Solution 3 (Flow Rate - 1.4 ml/min)

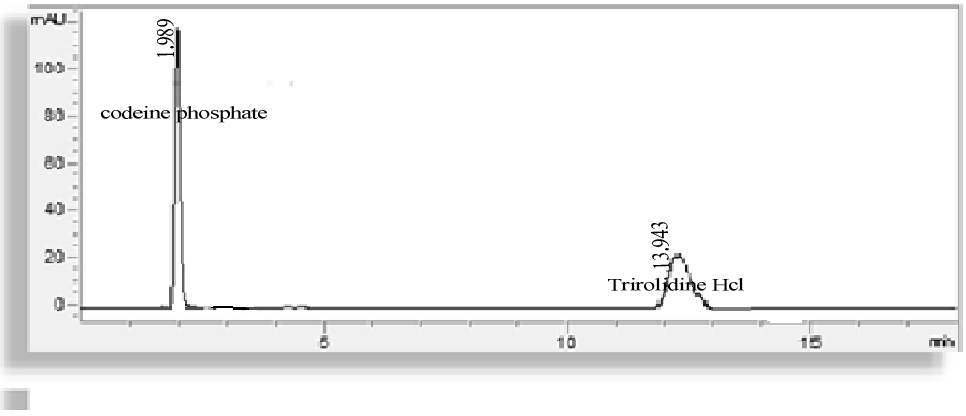


Table No. 45. Results of Robustness - Standard Solution 3

(Flow Rate - 1.4 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	1.989	825939	0.72	3897
Triprolidine hydrochloride	13.943	624786	0.61	4878

Figure No. 50. Chromatogram of Robustness - Standard Solution 4

(Flow Rate - 1.4 ml/min)

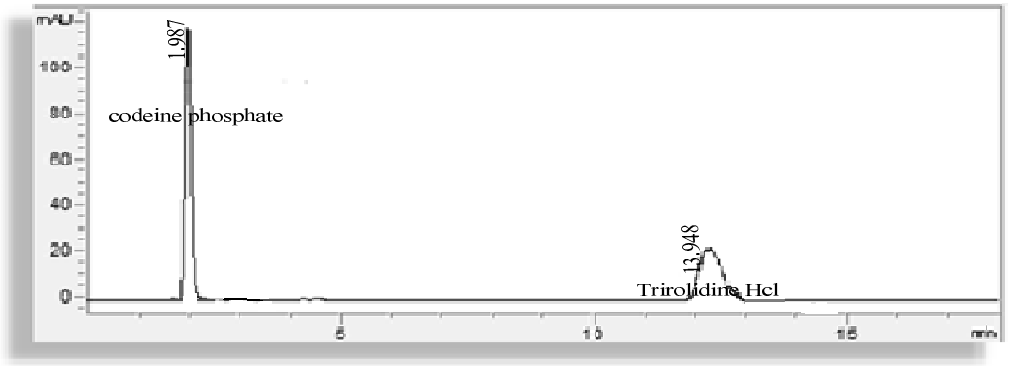


Table No. 46. Results of Robustness - Standard Solution 4

(Flow Rate - 1.4 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	1.987	825330	0.74	3896
Triprolidine hydrochloride	13.948	624282	0.60	4862

Figure No. 51. Chromatogram of Robustness - Standard Solution 5 (Flow Rate - 1.4 ml/min)

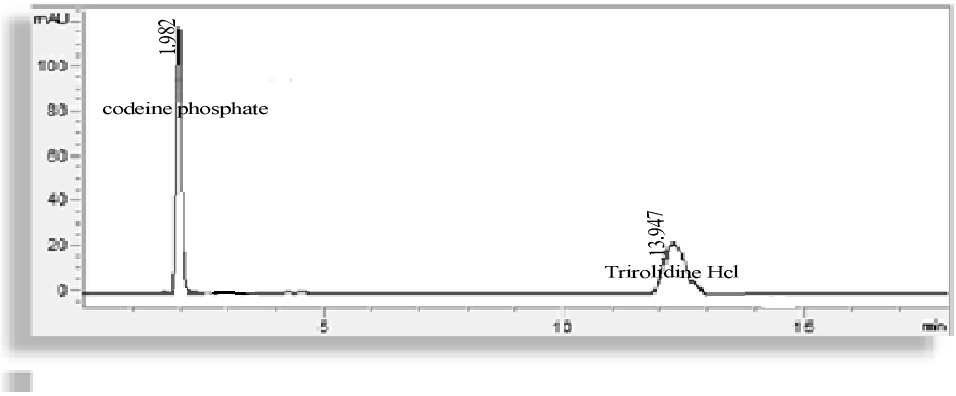


Table No. 47. Results of Robustness - Standard Solution 5 (Flow Rate - 1.4 ml/min)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	1.982	825934	0.76	3894
Triprolidine hydrochloride	13.947	624383	0.60	4871

iii) pH Variation - 1 (2.2):

Figure No. 52. Chromatogram of Robustness - Standard Solution (pH - 2.2)

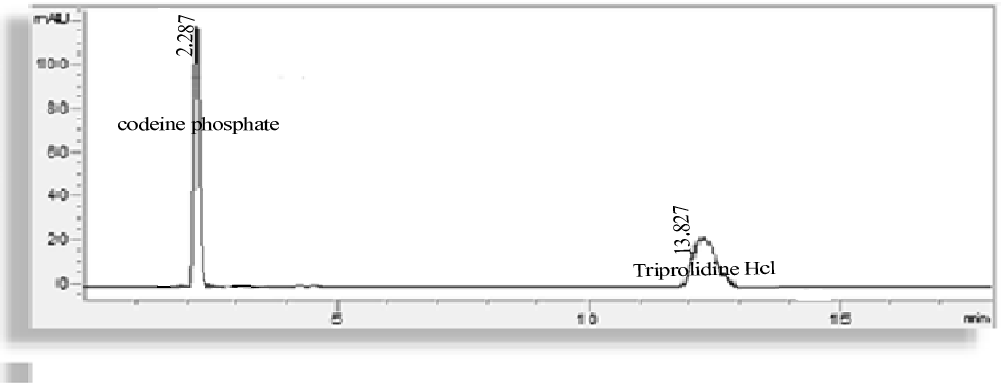


Table No. 48. Results of Robustness - Standard Solution (pH - 2.2)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.287	865928	0.74	3799
Triprolidine hydrochloride	13.827	604285	0.65	4859

iv) pH Variation - 2 (2.4):

Figure No. 53. Chromatogram of Robustness - Standard Solution (pH - 2.4)

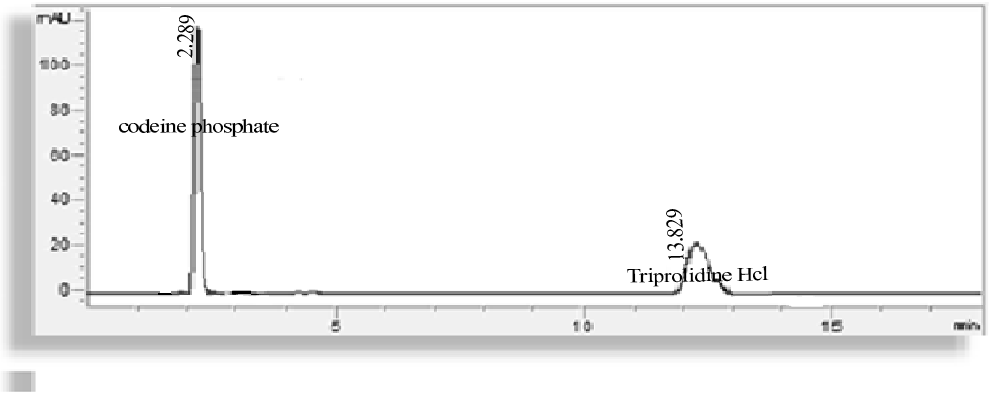


Table No. 49. Results of Robustness - Standard Solution (pH - 2.4)

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.289	865924	0.73	3795
Triprolidine hydrochloride	13.829	604287	0.64	4855

Table No. 50. Results of Robustness - Flow Rate Variation/Standard Solution 1 to 5 (Overall Mean, SD, % RSD for Codeine phosphate and Triprolidine hydrochloride)

Flow Rate Variation		RT	Area	Tailing Factor	Theoretical Plates
Flow Rate - 1.2 ml/min					
Codeine phosphate	**Mean**	2.2886	921906.8	0.726	3780.2
	SD	0.0062	24.503	0.0114	37.7001
	% RSD	0.2	0.24	1.5	1.0
Triprolidine hydrochloride	**Mean**	13.9296	1303.842	0.63	4865
	SD	0.0079	614898	0.0081	3.0822
	% RSD	0.05	0.21	1.2	0.06
Flow Rate - 1.4 ml/min					
Codeine phosphate	**Mean**	1.9854	826574	0.738	3896
	SD	0.0026	1893.43	0.0148	2.1213
	% RSD	0.13	0.22	2.0	0.05
Triprolidine hydrochloride	**Mean**	13.9464	624465.4	0.604	4869
	SD	0.0021	217.43	0.0054	6.1886
	% RSD	0.017	0.03	0.9	0.12

8.7 Ruggedness:

Figure No. 54. Chromatogram of Ruggedness (Blank)

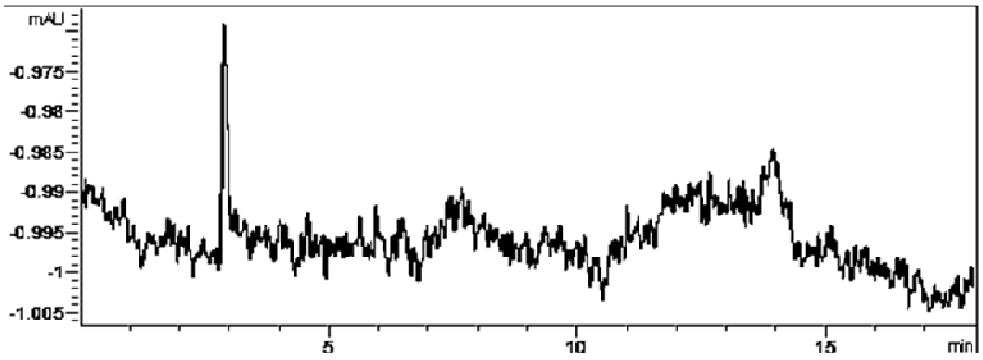


Figure No. 55. Chromatogram of Ruggedness - Day 1 (Analyst - 1) for Codeine phosphate and Triprolidine hydrochloride

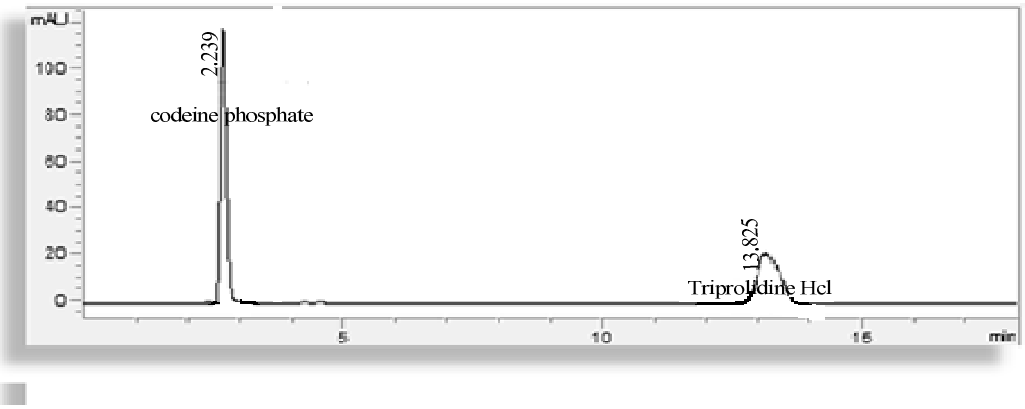


Table No. 51. Results of Ruggedness - Day 1 (Analyst - 1) for Codeine phosphate and Triprolidine hydrochloride

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.239	816151	0.73	3795
Triprolidine hydrochloride	13.825	623254	0.64	4863

Figure No. 56. Chromatogram of Ruggedness - Day 2 (Analyst - 2) for Codeine phosphate and Triprolidine hydrochloride

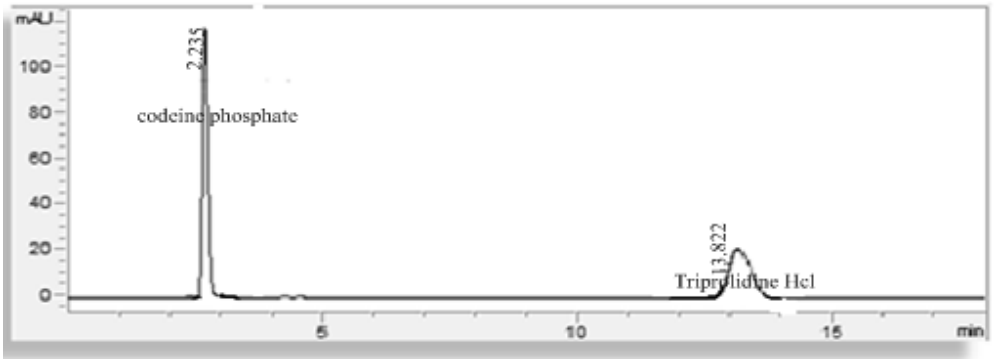


Table No. 52. Results of Ruggedness - Day 2 (Analyst - 2) for Codeine phosphate and Triprolidine hydrochloride

Name	RT	Area	Tailing Factor	Theoretical Plates
Codeine phosphate	2.235	816152	0.75	3794
Triprolidine hydrochloride	13.822	623254	0.63	4861

Table No. 53. Results of Ruggedness - Day 1 & 2 (Analyst - 1) for Codeine phosphate and Triprolidine hydrochloride

Analyst - 1				
S. No.	**Day - 1**		**Day - 2**	
	Codeine phosphate	**Triprolidine hydrochloride**	**Codeine phosphate**	**Triprolidine hydrochloride**
1	816151	623254	824035	625842
2	818636	624792	822456	624689
3	816152	624434	826945	623688
Mean	816979.6	624160	824478.6	624739.6
SD	2028.58	1138.12	3220.37	1524.37
% RSD	0.24	0.1	0.3	0.2

Table No. 54. Results of Ruggedness - Day 1 & 2 (Analyst - 2) for Codeine phosphate and Triprolidine hydrochloride

Analyst - 2				
S. No.	**Day - 1**		**Day - 2**	
	Codeine phosphate	**Triprolidine hydrochloride**	**Codeine phosphate**	**Triprolidine hydrochloride**
1	820077	624039	816152	623254
2	827463	624578	827628	628846
3	824628	624158	821968	626904
Mean	824056	624258.3	821916	62634.6
SD	3456.97	400.45	8115.00	4015.15
% RSD	0.4	0.06	0.9	0.6

CHAPTER - IX

SUMMARY AND CONCLUSION

- Development of new analytical methods for the determination of drugs in pharmaceutical dosage forms is more important in pharmacokinetic, toxicological and biological studies. Today pharmaceutical analysis entails much more than the analysis of active pharmaceutical ingredients or the formulated product. The pharmaceutical industry is under increased scrutiny from the government and the public interested groups to contain costs and at consistently deliver to market safe, efficacious product that fulfill unmet medical needs.
- The pharmaceutical analyst plays a major role in assuring identity, safety, efficacy, purity and quality of a drug product. The need for pharmaceutical analysis is driven largely by regulatory requirements. The commonly used tests of pharmaceutical analysis generally entail compendia testing method development, setting specifications and method validation.
- Analytical testing is one of the more interesting ways for scientists to take part in quality process by providing actual data on the identity, content and purity of the drug products. New methods are now being developed with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.
- In the present study a new RP-HPLC method was developed for the simultaneous determination of Codeine phosphate (CP) and Triprolidine hydrochloride (TH) in cough syrup formulation. A sample preparation is very simple and the analysis time is short.

- ❖ The analysis is resolved by using an Inersustain C18 (250 x 4.6 mm, 5µ) column, in isocratic mode, with mobile phase containing buffer (pH 3.0 with Orthophosphoric acid) and methanol in the final ratio of 55:45 v/v was used. The flow rate was 1.0 ml/min and the analyte was monitored at 280 nm. The retention time for Codeine phosphate and Triprolidine hydrochloride were about 2.8 min. and 12.4 min. respectively.
- ➢ The method was validated (As per ICH guidelines) for System suitability, Accuracy, Precision, Specificity, Linearity, Robustness and Ruggedness. The overall validation summary was tabulated as shown in **Table No. 55**.
  - ★ ***System Suitability:*** From the observation it was concluded that the method passes system suitability.
  - ★ ***Accuracy (Recovery Studies):*** The % recovery results were found with in the limits. It was concluded that the test method has an acceptable level of accuracy from 50% to 150% of target concentration.
  - ★ ***Precision:*** Test results for Codeine phosphate and Triprolidine hydrochloride were shown that the % RSD of peak area was found within the limits. Hence, the method was precise.
  - ★ ***Specificity:*** It was concluded that there is no interference due to blank and placebo solution. Hence the method is specific and the results were found to be complying with the acceptance criteria.
  - ★ ***Linearity:*** The correlation coefficient values were found within the acceptance limits.
  - ★ ***Robustness:*** The effect of change in the flow rate and pH of buffer solution in mobile phase, it was concluded that the % RSD of peak area was found within the limits. Hence, the method was robust.

★ ***Ruggedness:*** The % RSD of peak area was found within the limits. It was concluded that the test method is rugged.

Table No. 55. Validation Summary

S. No.	Parameter	Experiment	Acceptance Criteria	Observed Results	
				CP	TH
1	System Suitability	Peak area	❖ % RSD of peak area should NMT 2.0.	0.04	0.11
		Tailing factor	❖ The tailing factor for CP and TH should NMT 2.0.	1.9	1.8
		Theoretical plates	❖ The column efficiency for CP and TH should NLT 3000 theoretical plates.	3856	5186
2	Accuracy	% recovery	❖ % recovery in all the cases should be between 100±2.	99.8 %	100 %
3	Precision	Peak area	❖ % RSD of peak area should NMT 1.0.	0.0003 %	0.0003 %
4	Specificity	Interference	❖ There should not be any interference in the blank and placebo solution.	No interference	No interference

5	Linearity	Correlation coefficient	❖ Correlation coefficient should NLT 0.995.	0.999	0.999
6	Robustness	Peak area **Flow rate variation:** Flow Rate - 1.2 ml/min Flow Rate - 1.4 ml/min	❖ % RSD of peak area should NMT 1.0.	 0.24 0.22	 0.21 0.03
7	Ruggedness	Peak area **Analyst 1:** Day - 1 Day - 2 **Analyst 2:** Day - 1 Day - 2	❖ % RSD of peak area should NMT 1.0.	 0.24 0.3 0.4 0.9	 0.1 0.2 0.06 0.6

❖ Finally it was concluded that the proposed method are sensitive, accurate, precise, simple and reproducible and can be used for the simultaneous determination of Codeine phosphate and Triprolidine hydrochloride in cough syrup formulation by RP-HPLC.

CHAPTER - X

BIBLIOGRAPHY

1. Sethi, PD., HPLC Quantitative Analysis of Drugs in Pharmaceutical Formulations, 1st Edn., CBS Publishers and Distributors, New delhi, 2001, 94-105.
2. Snyder, L.R., Kirkland, J.J., Glajch, J.L., Practical HPLC Method Development, 2nd Edn., John Wiley and Sons, New York, 1999, 234-260.
3. Ashutosh Kar, Pharmaceutical Drug Analysis, 2nd Edn., New Age International Private Limited, 2005, 452-474.
4. https://www.etymonline.com/word/chromatography, "Chromatography". Online Etymology Dictionary and http://en.wikipedia.org/wiki/chromatography.
5. Chatwal, G.R., Anand, S.K., Instrumental Methods of Chemical Analysis, 5th Edn., Himalaya Publishing House Pvt Ltd., 2016, 1.2-1.5.
6. Sharma, BK., Instrumental Methods of Chemical Analysis, 23rd Edn., Goel Publishing House, Meerut, 2004, 7-10.
7. RamaRao, G., Murthy, S.S.N., Khadgapathi, P., High Performance Liquid Chromatography and its role in Pharmaceutical Analysis, Eastern Pharmacist, 1986, 20 (346), 53-59.
8. Sastry, C.S.P., Prasad, T.N.V., Rao, E.V., Recent Application of High Performance Liquid Chromatography in Pharmaceutical Analysis, Indian. Pharm. Education., 1987, 21, 37-40.
9. Willard, H.H., Merritt, L.L., Dean, J.A., Settle, F.A., Instrumental Methods of Analysis, 7th Edn., CBS Publishers and Distributors, New Delhi, 1991, 436-439.

10. Snyder, L.R., Kirkland, J.J., Glajch, J.L., Practical HPLC Method Development, 2nd Edn., John Wiley and Sons, New York, 1999, 742-747.
11. International Conference on Harmonization (ICH)-Validation of Analytical Procedures: Methodology (Q2B), Food and Drug Administration, USA, 1996.
12. International Conference on Harmonization (ICH)-Text on Validation of Analytical Procedures (Q2A), Geneva, 1994, 1-5.
13. http: //en. Wikipedia. Org/ wiki/ codeine phosphate.
14. http: //en. Wikipedia. Org / wiki / triprolidine hydrochloride.
15. Manassra, A., Khamis, M., El-Dakiky, M., Abdel-Qader, Z., Al-Rimawi, F., Simultaneous HPLC Analysis of Pseudophedrine hydrochloride, Codeine phosphate and Triprolidine hydrochloride in Liquid Dosage Forms, J. Pharm. Biomed. Anal., 2010, 51(4), 991-993.
16. Capella-Peiró, M.E., Bose, D., Rubert, M.F., Esteve-Romero, J., Optimization of a Capillary Zone Electrophoresis Method by Using a Central Composite Factorial Design for the Determination of Codeine and Paracetamol in Pharmaceuticals, Journal of Chromatography B, 2006, 839(1-2), 95-101.
17. Gomez, M.R., Sombra, L., Olsina, R.A., Martínez, L.D., Silva, M.F., Development and Validation of a Capillary Electrophoresis Method for the Determination of Codeine, Diphenhydramine, Ephedrine and Noscapine in Pharmaceuticals, Il Farmaco, 2005, 60(1), 85-90.
18. Kartal, M., LC Method for the Analysis of Paracetamol, Caffeine and Codeine phosphate in Pharmaceutical Preparations, Journal of Pharmaceutical and Biomedical Analysis, 2001, 26(5-6), 857-864.
19. Hood, D.J., Cheung, H.Y., A Chromatographic Method for Rapid and Simultaneous Analysis of Codeine phosphate, Ephedrine HCl and

Chlorpheniramine maleate in Cough-Cold Syrup Formulation, Journal of Pharmaceutical and Biomedical Analysis, 2003, 30(5), 1595-1601.

20. Ragonese, R., Mulholland, M., Kalman, J., Full and Fractionated Experimental Designs for Robustness Testing in the High-Performance Liquid Chromatographic Analysis of Codeine phosphate, Pseudoephedrine hydrochloride and Chlorpheniramine maleate in a Pharmaceutical Preparation, Journal of Chromatography A, 2000, 870(1-2), 45-51.
21. Lau, I.W., Mok, C.S., High-Performance Liquid Chromatographic Determination of Active Ingredients in Cough-Cold Syrups with Indirect Conductometric Detection, Journal of Chromatography A, 1995, 693(1), 45-54.
22. Weingarten, B., Wang, H.Y., Roberts, D.M., Determination of Codeine in Human Plasma by High-Performance Liquid Chromatography with Fluorescence Detection, Journal of Chromatography A, 1995, 696(1), 83-92.
23. Santoni, G., Fabbri, L., Gratteri, P., Renzi, G., Pinzauti, S., Simultaneous Determination of Aspirin, Codeine phosphate and Propylphenazone in Tablets by Reversed-Phase High Performance Liquid Chromatography, International Journal of Pharmaceutics, 1992, 80(1-3), 263-266.
24. Chen, Z.R., Bochner, F., Somogyi, A., Simultaneous Determination of Codeine, Norcodeine and Morphine in Biological Fluids by High-Performance Liquid Chromatography with Fluorescence Detection, Journal of Chromatography B: Biomedical Sciences and Applications, 1989, 491, 367-378.
25. Ginman, R., Thomas Karnes, H., Perrin, J., Simultaneous Determination of Codeine and Ibuprofen in Plasma by High Performance Liquid Chromatography, Journal of Pharmaceutical and Biomedical Analysis, 1985, 3(5), 439-445.

26. Davidson, A.G., Mkoji, L.M.M., The Simultaneous Assay of Triprolidine, Psudoephedrine and Dextromethorphan in Combined Preparations by Derivative-Difference Spectrophotometry, Journal of Pharmaceutical and Biomedical Analysis, 1988, 6(5), 449-460.
27. El-Gindy, A., Abdel-Salam Attia, K., Nassar, M.W., Abu Seada, H.H., Al-Shabrawi Shoeib, M., HPLC Method for Determination of Paracetamol, Psudoephedrine, Triprolidine, Methylparaben, Propylparaben, Sodium benzoate and their Related Substances in Pharmaceutical Syrup, Journal of Liquid Chromatography & Related Technologies, 2013, 36(9), 1251-1263.
28. Sriphong, L., Chaidedgumjorn, A., Chaisuroj, K., Derivative Spectrophotometry Applied to the Determination of Triprolidine hydrochloride and Pseudoephedrine hydrochloride in Tablets and Dissolution Testing, World Academy of Science, Engineering and Technology, 2009, 31, 569-573.
29. Caglar, H., Buyuktuncel, E., HPLC Method Development and Validation:Simultaneous Determination of Active Ingredients in Cough and Cold Pharmaceuticals, International Journal of Pharmacy and Pharmaceutical Sciences, 2014, 6(10), 421-428.
30. De Orsi, D., Gagliardi, L., Balasco, A., Tonelli, D., Simultaneous Determination of Triprolidine, Pseudoephedrine, Paracetamol and Dextromethorphan by HPLC, Chromatographia, 1996, 43(9-10), 496-500.
31. Mallu, U.R., Bobbarala, V., Penumajji, S., Analysis of Cough and Analgesic Range of Pharmaceutical Active Ingredients using RP-HPLC Method, International Journal of Pharma and Bio Sciences, 2011, 2(3), 439-452.
32. Paidipala, K., Kamarapu, S.K., Method Development and Validation of RP-HPLC Method for Simultaneous Estimation of Dextromethorphan

hydrobromide, Phenylephrine hydrochloride and Triprolidine hydrochloride in Bulk and Combined Tablets Dosage Forms, International Journal of Pharmacy and Biological Sciences, 2013, 3(3), 172-179.

33. Hinge, M., Patel, K.R., Mahida, R.J., Spectrophotometric and High Performance Liquid Chromatographic Determination (HPLC) of Triprolidine and Pseudoephedrine hydrochloride in Tablet Dosage Form, Pharmaceutical Methods, 2015, 6(2), 87-93.
34. Abu Reid, I.O., Awad Widaa, T., Spectrophotometric Method for the Simultaneous Determination of Pseudoephedrine and Triprolidine in Bulk and Tablet Forms, International Journal of Advances in Pharmaceutical Analysis, 2017, 07 (03), 21-23.
35. Arif Aziz, A., Ahmed, K., Simultaneous Determination of Paracetamol with Different Active Pharmaceutical Ingredient (API) and Excipient in Various Dosage Forms, Sci. Int. (Lahore), 2015, 27(6), 6173-6176.
36. Ma, D., Du, J., Liu, M., Determination of Triprolidine hydrochloride in Capsules and Binding Study of Triprolidine hydrochloride to Serum Albumins with Chemiluminescence Method, Spectroscopy Letters, 2015, 48(5), 343-350.
37. Vijai Anand, P.R., Deepika, N., Sam Solomon, W.D., Venkatanarayanan, R., RP-HPLC Method Development and Validation for Simultaneous Determination of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in Cough Syrup Formulation, Journal of Pharmacy Research, 2012, 5(2), 949-953.
38. Kazemi, M.S., Boorumand, S., Moosavi Asad, S.M., Extraction and Determination of Codeine phosphate in Water Samples by Dispersive Liquid-Liquid Microextraction Coupled to UV-Vis Spectrophotometry, Der Pharma Chemica, 2015, 7(1), 277-282.

39. Geetha Lakshmi, E., Ramasamy, M., A Novel RP-HPLC Method for Simultaneous Estimation of Codeine phosphate, Chlorpheniramine maleate and its Preservative in Syrup Formulation, International Journal of Pharmacy and Pharmaceutical Sciences, 2012, 4(3), 585-591.
40. Nazir, A., Naseer, Y., Shahid, R., Raza, S., Development & Validation of Analytical Method Used for Simultaneous Determination of Paracetamol, Caffeine and Codeine phosphate by HPLC in Pharmaceutical Formulation, Sci. Int. (Lahore), 2016, 28(3), 2497-2499.

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